BULLETIN OF THE
AGRICULTURAL CHEMICAL SOCIETY
OF JAPAN

CONTENTS

The Bulletin of the Agricultural Chemical Society of Japan is published bimonthly to provide original papers on agricultural chemistry.

Manuscripts submitted for publication and all inquiries regarding them should be sent to the Editor of the Bulletin, The Agricultural Chemical Society of Japan, c/o Departments of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Annual subscription price is $ 6.00 for all oversea subscribers including postage fee. Please address orders to the sole agent.

The Agricultural Chemical Society of Japan also published the Journal of the Agricultural Chemical Society of Japan (Nippon Nogei Kagakukaishi), printed in Japanese.

Studies on Osmophilic Yeasts

Part I.   Salt-tolerance and Sugar-tolerance of Osmophilic Soy-yeasts

By Hiroshi Ōnishi

Noda Institute for Scientific Research

The characteristic of salt-tolerance of the osmophilic soy yeasts was different from their sugar-tolerance in the points of viability of the individual cells in a high concentration of salts or sugars of the same osmotic pressure, and of the limiting osmotic pressure for their growth.   It became much more apparent that growth of the osmophilic yeasts in the medium of a high concentration of sodium chloride involved a process of physiological adaptation. The order of the toxicity of the salts for the growth of *Zygosaccharomyces major* was as follows :
$$K^+ < Na^+ < Mg^{++} < Ca^{++} < Li^+ \qquad Cl^- < SO_4^{--} < NO_3^-$$

In the previous report[1], it was found that in the viable counts of the yeasts grown in soy sauce mashes, which differed significantly from other brewage mashes in their high contents of sodium chloride (NaCl 18%) and nitrogenous compounds (as total N, 1.0~1.5%), a remarkable fall was observed in a single step transplantation when ordinary NaCl-free media were used for plating count, while there was not any significant fall to be observed when media containing sodium chloride in the same concentrations as soy sauce mashes were used, and also a fall in viability was similarly observed when, in a reverse direction, these yeasts grown in the plain medium (NaCl 0%) were directly transferred to the saline medium (NaCl 18%).   Since the osmophilic property of soy yeasts against sodium chloride could be increased by culturing in media containing sodium chloride, the author has regarded this, to a certain extent, as an adaptive nature, though this property had hitherto been simply believed to be genetically constant and transmissible.

From the above facts, it seemed that fundamental studies on the influences of sodium chloride on growth and metabolism of the osmophilic yeasts should be carried on further, and elucidation of these studies might be of great interest in the manufacture of soy sauce and miso paste, and furthermore in the preservation of salted food.

In this paper, the characteristics of salt-tolerance and sugar-tolerance of osmophilic soy yeasts are shown.

EXPERIMENTAL

(1) Reversibility of Salt-tolerance of Osmophilic Soy Yeasts.   The stability of NaCl-tolerance which was acquired by growing in a medium containing sodium chloride was examined.   Three strains of soy yeasts, *Zygosaccharomyces major*, N 5 and N 8 were cultured successively in NaCl-free and NaCl 18% koji extract media on the schedule shown in Table I.   The salt-tolerance of the yeast cells grown in each cultural environment was measured by plating them on NaCl-free and NaCl 18% koji extract agar.   As shown in Table I, salt- tolerance which was acquired by the yeast cells grown in a NaCl 18% medium, was easily lost if these yeasts were once cultured in a NaCl-free medium.   Accordingly, even in case of the osmophilic yeasts, salt-tolernance was found to be much dependable on their cultural conditions, i.e. the presence and absence of NaCl.

(2) Growth of Soy Yeasts on Various Media of Different Concentrations of Sodium Chloride.   As shown in Table II, when the yeasts cultured in

(1) H. Onishi, *J. Agr. Chem Soc. Japan*, **28**, 546 (1954).

TABLE I

REVERSIBILITY OF SALT-TOLERANCE OF OSMOPHILIC YEASTS DEPENDING
UPON THEIR CULTURAL ENVIRONMENTS

Cultural sequence: (Liquid culture in koji extract)

```
                                              ┌──→ NaCl  0% (IIIa)
                            ┌→ NaCl 0% (IIa) ─┤
                            │                 └──→ NaCl 18% (IIIb)
NaCl 0%──→NaCl 18% (I) ─────┤
                            │                 ┌→ NaCl  0% (IIIc)
                            └→ NaCl 18% (IIb) ─┤
                                              └→ NaCl 18% (IIId)
```
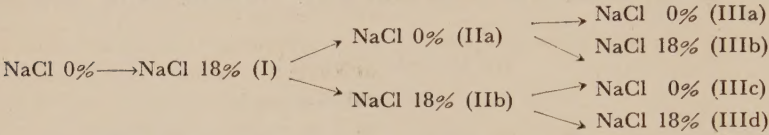
Strains	NaCl % of plating media	Cultural environments						
		I	IIa	IIb	IIIa	IIIb	IIIc	IIId
Z. major {	0	80*	396	3	572	33	352	82
	18	1456	10	952	45	250	35	248
N 8 {	0	110	96	53	472	35	528	32
	18	1310	18	976	62	169	90	676
N 5 {	0	15	152	9	508	155	712	162
	18	816	10	412	20	1778	71	1704

* These values indicate the number of colonies on each plate.

TABLE II

CHANGE IN VIABILITY OF YEAST CELLS WHEN TRANSFERRED FROM A NaCl-FREE
MEDIUM TO MEDIA OF VARIOUS CONCENTRATIONS OF NaCl

Strains	NaCl % of Plating media (koji extract agar)				
	0%	5%	10%	15%	18%
Z. major	936	912	728	372	108
Z. soya	284	260	194	24	5
A 31	538	508	436	276	170
A 34	158	173	166	71	31
N 5	140	154	149	49	14
N 8	342	376	286	123	35
N 15	310	290	266	72	21
N 21	340	340	364	248	70
N 24	928	896	880	728	404
N 28	128	153	136	63	36

Tested strains were isolaed from soy sauce mashes and showed high salt-tolerance.

TABLE III

CHANGE IN VIABILITY OF YEAST CELLS WHEN TRANSFERRED FROM NaCl
5% TO NaCl 18% MEDIUM

Strains	NaCl % of plating medium		Strains	NaCl % of plating medinm	
	5%	18%		5%	18%
Z. major	2192	1584	N 8	856	812
Z. soya	404	364	N 15	968	712
A 31	1704	1480	N 21	1872	1832
A 34	808	760	N 24	776	800
N 5	1352	1168	N 28	584	546

NaCl-free medium were transferred in a single step to the media of various NaCl concentrations, the fall in viability was very small up to 10% but remarkable above 15% of NaCl in the medium.

It is noteworthy that when the yeasts cultured in the medium of relatively low concentrations such as 5% of NaCl were transferred to the NaCl 18% medium, the fall in viability was remarkably small.

(Table III)

The growth curve in the NaCl 18% medium, manifested by *Z. major* which was precultured in the media of different NaCl concentrations as 0, 5, 10 and 18% is shown in Fig. 1.

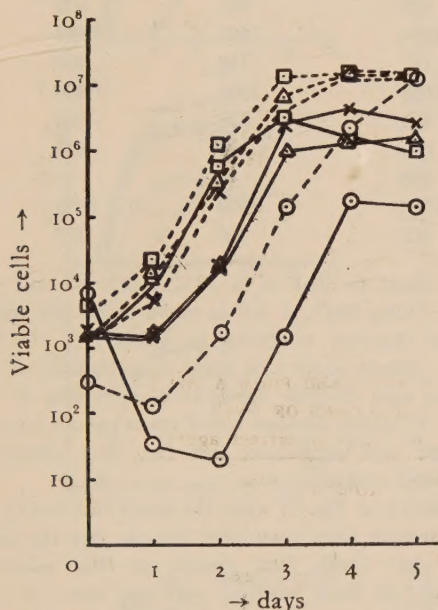The composition of the liquid medium employed

FIG. 1. The Effects of the Precultural Conditions on the Growth of *Z. major* in NaCl 18% Medium.

○ : precultured in NaCl-free medium
× : precultured in NaCl 5% medium
△ : precultured in NaCl 10% medium
□ : precultured in NaCl 18% medium
solid line : plating on NaCl-free medium
broken line : plating on NaCl 18% medium

was as follows: glucose 5%; KH_2PO_4 0.1%; $MgSO_4 \cdot 7H_2O$ 0.05%; $CaCl_2 \cdot 2H_2O$ 0.01%; NaCl 0.01%; Bacto vitamin-free casamino acid 0.03% as total nitrogen; Bacto yeast extract 0.2%; thereto NaCl was added to the required amount.

The growth of yeast was measured by a plating count using NaCl-free and NaCl 18% media.

As shown in Fig. 1, when yeasts precultured in the medium containing more than 5% NaCl were inoculated in the NaCl 18% medium, the fall in viability at the lag phase of growth was not observed, but when precultured in the NaCl-free medium, the fall in viability at the beginning of the growth curve was remarkable.

From these facts, it became much more clear that the growth of osmophilic yeasts in the medium of a high concentration of sodium chloride involves a process of physiological adaptation, this not merely being a recovery of yeast cells from the osmotic effects produced by sodium chloride.

(3) Limiting Osmotic Pressure for the Growth of Various Yeasts. Limit concentrations of sodium chloride and sugars for the growth of ordinary yeasts such as *Saccharomyces sake* and *Sacch. ellipsoideus* were found to be 10% in NaCl, 50% in glucose and 60% in saccharose, and for osmophilic yeasts, such as *Zygosaccharomyces major* and *Zygosacch. soya* were 20~22% in NaCl, 80% in glucose and 80% in saccharose, respectively.

Osmotic pressure of the medium was calculated from the formula of Lewis[2].

$$II = 12.06\varDelta - 0.021\varDelta^2$$

where

II : osmotic pressure (atm.)

$\varDelta$: degree of freezing point depression (°C)

Values for freezing point depression of NaCl solution were taken from the Table of Landolt-Börnstein[3]. Published data on these measurements by Frazer and Myrick[4], Church[5] and International Critical Tables vol. 4 were also referred.

Limiting osmotic pressure for their growth was, therefore, calculated as 75 atm. in NaCl, 140 atm. in glucose and 90 atm. in saccharose for *Sacch. sake* and *Sacch. ellipsoidius*, and 150~165 atm. in NaCl, 220~260 atm. in glucose and 180~200 atm. in saccharose for *Zygosacch. major* and *Zygosacch. soya*.

It seemed quite natural that the soy yeasts, as a group of the so-called osmophilic yeasts show as above, much higher tolerance towards a high osmotic pressure than the ordinary yeasts; it was also noticeable that in general, the osmotic pressure which limits growth of the yeasts is much lower in NaCl than in sugar.

(4) The Growth of Soy Yeasts on the Media of High Concentrations of Sugar. Sugar concentration which corresponds to the osmotic pressure of a 18% NaCl solution (135 atm.) is 50% in glucose, 75% in saccharose and 50% in galactose respectively. Though a fall in viability was, as above-stated, very remarkable when soy yeasts cultured in NaCl-free

2) G.N. Lewis, *J. Am. Chem. Soc.* **30**, 668 (1908).

3) Landolt-Börnstein, *Physikalisch-Chemische Tabellen* **2**, 1452 (1923).

4) J.C.W. Frazer and R.T. Myrick, *J. Am. Chem. Soc.* **38**, 1907 (1916).

5) M.B. Church, *Science*, **74**, 492 (1931).

TABLE IV

CHANGES IN VIABILITY OF YEAST CELLS TRANSFERRED FROM THE PLAIN MEDIUM
TO MEDIA OF HIGH SUGAR CONCENTRATIONS

Strains	koji-agar	glucose 50% koji-agar	koji-agar	saccharose 75% koji-agar	koji-agar	galactose 50% koji-agar
Z. major	532	524	468	460	1512	1496
Z. soya	292	302	304	328	832	800
A 31	486	464	1232	1304	1600	1632
A 34	132	144	176	182	456	460
N 5	352	332	660	688	1000	896
N 8	364	366	560	564	828	828
N 15	115	122	772	832	400	444
N 21	544	536	532	568	—	—
N 24	342	308	1624	1560	1568	1656
N 28	158	175	328	372	206	202

medium were transplanted into the NaCl 18% medium, it was examined whether a similar change in viability would really occur when in place of NaCl-medium, a sugar medium, whose osmotic pressure was adjusted

FIG. 2. Growth Curve of Z. major in the high Sugar Concentration Medium.

    O : inoculated into the basal plain medium
    × : inoculated into the glucose 50% medium
    △ : inoculated into the galactose 50% medium
    □ : inoculated into the NaCl 18% medium
           ——— : plain koji agar
           - - - - : glucose 50% koji agar
           —·—· : NaCl 18% koji agar

to be equal to that of a 18% NaCl solution was used. Unlike NaCl, a fall in viability for the sugars was not observed as shown in Table IV. In this test, it cannot occur that sugar concentration of the medium may decrease significantly according to the growth of yeasts, because tested yeasts cannot ferment galactose and saccharose, but can only assimilate them very weakly[6].

As shown in Fig. 2, when the yeasts cultured in the plain medium were inoculated directly into the basal plain, 18% NaCl, 50% glucose or 50% galactose medium (the basal medium was the same as that shown in Fig. 1), not only in the basal plain medium but also in the glucose 50% and galactose 50% medium, the fall in viability at the lag phase of the growth curve was not found, but in 18% NaCl medium, a remarkable fall in viability was observed at the initial stage of growth.

Now, it is apparent that characteristics of salt-tolerance and sugar-tolerance of the osmophilic soy yeasts toward the medium of the same osmotic pressure, are quite different from each other, so some limiting factors other than the osmotic pressure itself are presumable to have influence on the salt-tolerance of the osmophilic yeasts.

(5) Growth of Soy Yeasts in the Medium of a High Concentration of Various Salts. The growth of Zygosaccharomyces major in the liquid media of various concentrations of sodium sulfate, sodium nitrate, potassium chloride, lithium chloride, calcium chloride and magnesium sulfate was observed. The basal medium was the same as that shown in Figs. 1 and 2. The growth curve is shown

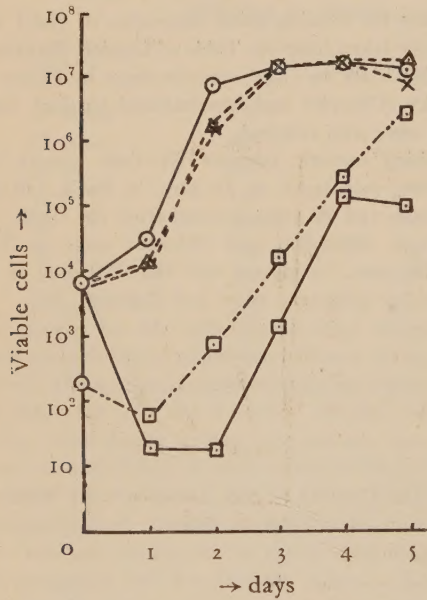6) H. Onishi, This bulletin (in press).
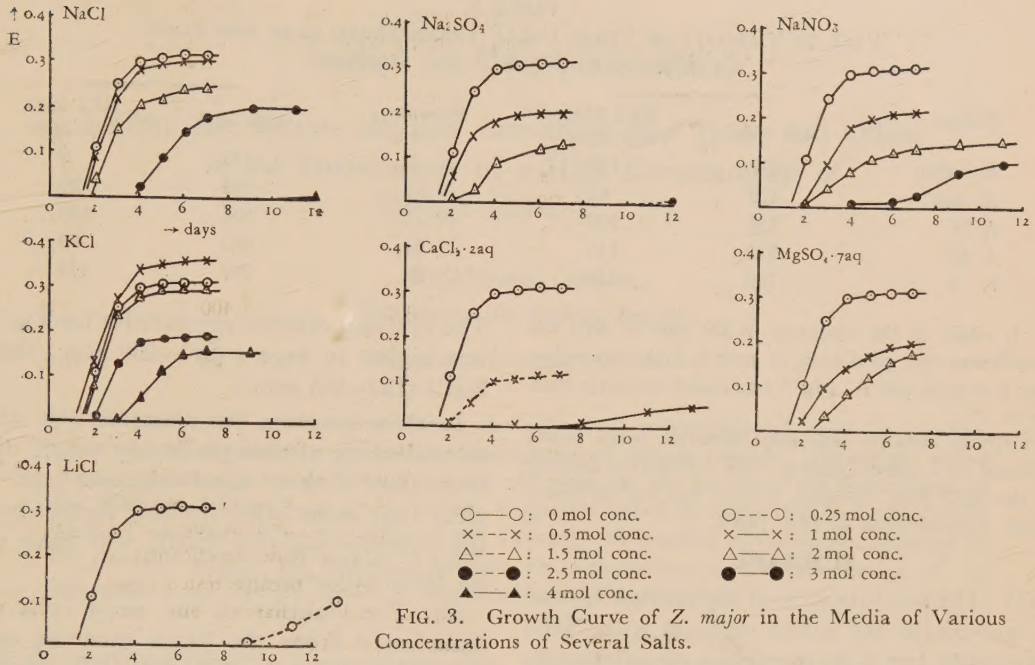
FIG. 3. Growth Curve of Z. *major* in the Media of Various Concentrations of Several Salts.

O——O : 0 mol conc.   O---O : 0.25 mol conc.
×---× : 0.5 mol conc.   ×——× : 1 mol conc.
△---△ : 1.5 mol conc.   △——△ : 2 mol conc.
●---● : 2.5 mol conc.   ●——● : 3 mol conc.
▲——▲ : 4 mol conc.

O : NaCl,   × : Na_2SO_4,   △ : $NaNO_3$,   ● : KCl
▲ : $MgSO_4 \cdot 7H_2O$,   ◑ : $CaCl_2 \cdot 2H_2O$,   □ : LiCl,
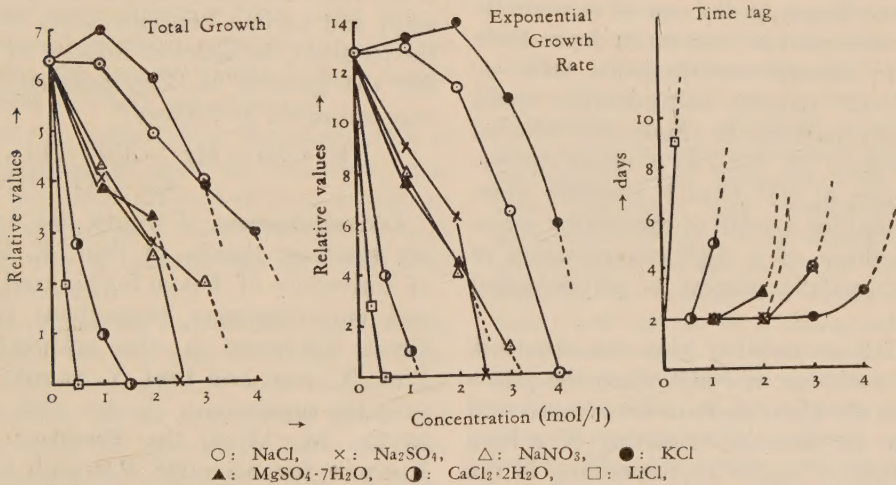
FIG. 4. Relations between the Growth Constant and the Concentrations of Several Salts for the Growth of Z. *major*.

in Fig. 3.

Relations between the growth constant, e.g. total growth, exponential growth rate and time lag, and the concentrations of salts in the medium are shown in Fig. 4. For *Zygosaccharomyces major*, the order of

toxicity of ions per molar concentration of the tested salts was as follows :

$$K^+ < Na^+ < Mg^{++} < Ca^{++} < Li^+, \quad Cl^- < SO_4^{--} < NO_3^-$$
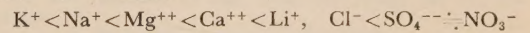
The growth of *Zygosacch. mojor* was inhibited by relatively low concentrations of LiCl such as 0.5 mol

TABLE V

FALL IN VIABILITY OF YEAST CELLS TRANSFERRED FROM THE PLAIN
MEDIUM TO THE KCl 23% MEDIUM

Strains	koji agar	KCl 23% koji agar	Strains	koji agar	KCl 23% koji agar
Z. *major*	1072	139	N 8	153	9
Z. *soya*	172	62	N 15	744	96
A 31	358	200	N 21	524	100
A 34	312	145	N 24	960	99
N 5	780	113	N 28	287	119

per 1, while on the contrary, in the case of KCl the growth was very good even in such a high concentration as 4 mols per 1. Mg^{++} was found less toxic than Ca^{++}.

A fall in viability was also observed when yeasts cultured in a plain medium were transferred directly to the KCl 23% medium which was of the same molar concentration as 18% NaCl.

SUMMARY

(1) The salt-tolerance of soy yeasts acquired by growth in the saline medium with NaCl was easily lost if these yeasts were afterwards once cultured in the ordinary medium without NaCl. Accordingly, in the case of osmophilic soy yeasts salt-tolerance was much dependable upon their cultural environments with or without NaCl. Extreme salt-tolerance could be acquired even when the yeasts were cultured in the medium of NaCl of a relatively low concentration as 5%. Thus it becomes quite apparent that the growth of osmophilic yeasts in the medium of a high concentration of NaCl involves the process of physiological adaptation.

(2) A fall in viability was not observed, contrary to the case of NaCl, when the yeasts cultured in the plain medium were transferred directly to the medium consisting of a high concentration of sugars corresponding to the osmotic pressure of 18% NaCl (135 atm.).

The limiting osmotic pressure for their growth was higher in sugars (220~260 atm.) than in NaCl (150~165 atm.).

In these respects, the characteristic of the salt-tolerance of osmophilic soy yeasts differs from that of their sugar-tolerance and it is clear that some other limiting factors besides the osmotic pressure itself, will have effect on their salt-tolerance.

(3) The viability of the yeast cells when transferred from the plain medium to the various salt media generally fell. Judging from the relations between the growth constant and various concentrations of salts in the medium, the order of toxicity of the salts for the growth of *Zygosaccharomyces major* was estimated as follows:
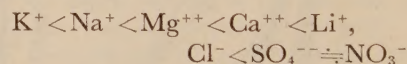
$$K^+ < Na^+ < Mg^{++} < Ca^{++} < Li^+,$$
$$Cl^- < SO_4^{--} \doteqdot NO_3^-$$

Studies on Osmophilic Yeasts

Part II. Factors affecting Growth of Soy Yeasts and Others in the Environment of a High Concentration of Sodium Chloride. (1)

By Hiroshi Ōnishi

Noda Institute for Scientific Research

The author has conducted studies on the nutritional requirement of yeasts in environments of both an ordinary and a high concentration of sodium chloride. During the course of these experiments, it was found that the pH range for the growth of the tested yeasts in the NaCl-free medium was in a very wide range (pH 3.0~7.0), while that in the NaCl 18% medium was limited in the range of pH 4.0~5.0. In addition to the results described in the previous paper,[1] the difference between the salt-tolerance and sugar-tolerance of the osmophilic yeasts was also shown in respect of this pH dependence.

In the previous report[1], it was shown that the properties of salt-tolerance of osmophilic soy yeasts were different from their sugar-tolerance and therefore some limiting factors other than the osmotic pressure itself, would have effect on their salt-tolerance.

In order to approach to the elucidation of the mechanism of their salt-tolerance, comparative studies on the growth in environments of both an ordinary (NaCl-free) and a high concentration of sodium chloride (NaCl 18%) were carried out.

In this paper, the nutritional requirements of nitrogen sources and vitamins in these comparative environments were studied and the influences of an appropriate pH of the medium upon the growth in both environmental conditions were also described.

EXPERIMENTAL

(1) Effects of Nitrogen Sources on the Growth of Osmophilic Yeasts in the Medium of a High Concentration of Sodium Chloride.

(a) Assimilation of Ammonium Nitrogen. It is well known that if an adequate supply of other nutrients is provided, the yeasts are able to utilize ammonium nitrogen such as ammonium sulfate as the sole source of nitrogen in an ordinary NaCl-free medium. As for the assimilation of N-compounds by yeasts, Schultz and Pomper[2] made a comparative investigation on the assimilation of amino acids by yeasts of various genera and species and stated that this property might be applied in the taxonomy of yeasts. Takahashi[3] made investigations of the requirements of vitamins in the assimilation of amino acids by yeasts and has pointed out that in such experiments, the inoculum size should be controlled under 5×10^3 cells per ml of cultural media. Besides these, many papers of Wickerham[4], Atkin[5], and Swanson and Lifton[6] were published. However, a comparative study on the requirement of nitrogen sources in two contrasting conditions, one of them an ordinary NaCl-free medium and the other NaCl in a concentration as high as 18% medium, has not yet been carried out. Some observations on such studies are shown below.

1) H. Onishi, This bulletin in press

2) A.S. Schultz and S. Pomper, *Arch. Biochem.* **19**, 184 (1948).
3) M. Takahashi, *J. Agr. Chem. Soc. Japan,* **28**, 395, 398 (1954).
4) L.J. Wickerham, *J. Bact.* **52**, 293 (1946).
5) L. Atkin, *Arch. Biochem.* **15**, 305 (1947).
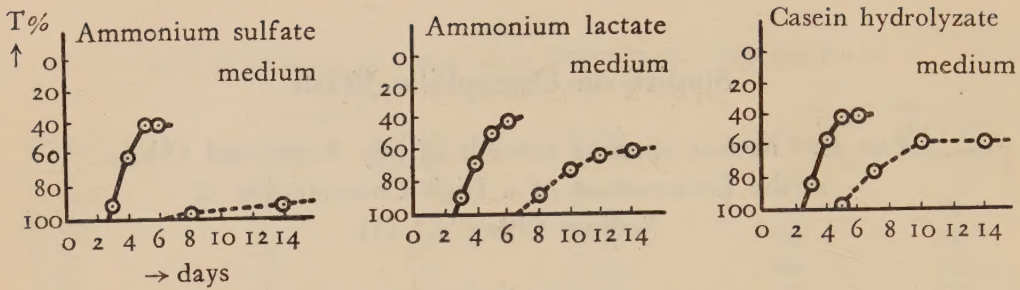6) H. Swanson and C. E. Lifton, *J. Bact.* **56**, 155 (1948).

FIG. 1. Growth Curve of Z. major in the Medium of Three Nitrogen Sources.
○——○: NaCl 0%,　　　　　○---○: NaCl 18%

Yeast strains: Z. major, Z. soya, Z. salsus, Z. miso α, S. miso α, Pichia miso, and nine strains which were isolated from soy sauce mashes by the author. These yeasts, of course, showed high salt-tolerance.

Media: As the basal medium, the modified Lodder and Kreger-van Rij's[7] medium was used. The composition of the medium was as follows: glucose 5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, vitamins (biotin 2 μg, Ca-pantothenate 400 μg, inositol 2000 μg, niacin 400 μg, p-amino benzoic acid 200 μg, pyridoxine 400 μg, thiamin 400 μg, riboflavin 200 μg, were added per 1 l of the medium). As a nitrogen source, each of the following ammonium sulfate, ammonium lactate and casein hydrolyzate was added to the basal medium in the amount of 0.03% as the total nitrogen. The following six media were tested. (1) ammonium sulfate medium, (2) NaCl 18% ammonium sulfate medium, (3) ammonium lactate medium, (4) NaCl 18% ammonium lactate medium, (5) casein hydrolyzate medium, (6) NaCl 18% casein hydrolyzate medium.

Methods of cultivation and observation: One loopful of the yeasts taken from the culture on koji extract agar at 30°C for 5~7 days was suspended in a sterilized physiological saline-solution, attention payed so as not to touch the agar layer. One drop of this suspension was inoculated to each medium and it was incubated at 30°C. In this case, size of the inoculum was less than 5×10^3 cells per ml of the media. The growth of yeasts was measured turbidimetrically with a photoelectric spectrophotometer at 660 mμ after 7 days' incubation in the NaCl-free and 14 days' incubation in the NaCl 18% medium. The results are shown in Table I and Fig. 1.

Table I indicates the extent of growth measured in values of light-transmittance % by the electrophoto-

7) J. Lodder and N. J. W. Kreger-van Rij, "The Yeasts a taxonomic study" p. 25 (1952).

TABLE 1

EFFECTS OF THE VARIOUS NITROGEN SOURCES ON THE GROWTH OF OSMOPHILIC YEASTS IN THE PLAIN (NaCl 0%) AND THE SALINE (NaCl 18%) MEDIA

Strains	Ammonium sulfate NaCl 0%	Ammonium sulfate NaCl 18%	Ammonium lactate NaCl 0%	Ammonium lactate NaCl 18%	Casein hydrolyzate NaCl 0%	Casein hydrolyzate NaCl 18%
Z. major	++	+	++	++	++	++
Z. soya	++	+	++	+	++	++
Z. salsus	++	+	++	++	++	++
Z. miso α	++	±	++	++	++	++
S. miso α	++	+	++	++	++	++
P. miso	+++	++	+++	++	+++	+++
N 3	++	±	++	++	++	++
N 5	++	+	++	++	++	++
A 6	++	++	+++	+++	+++	+++
N 8	++	+	++	++	++	++
N 15	++	+	++	++	++	+++
N 24	++	±	++	+++	++	+++
N 28	++	±	+++	+++	+++	+++
A 31	++	+	++	++	+++	++
A 34	++	+	++	++	+++	++

meter as follows: 100~98: −, 98~90: ±, 90~80: +, 80~70: ++, 70~50: +++, 50~0: +++.

From Table I and Fig. 1, it was found that in the NaCl-free medium, all strains could fully utilize all of the three nitrogen sources, but in the presence of 18% NaCl, assimilation of ammonium sulfate was apparently very poor. But this poor growth cannot be attributed to the low assimilability of ammonium

TABLE II

GROWTH OF OSMOPHILIC YEASTS IN THE AMMONIUM SULFATE MEDIUM ADDED WITH THE BUFFER

Strains	NaCl % of medium		Strains	NaCl % of medium		Strains	NaCl % of medium	
	0%	18%		0%	18%		0%	18%
Z. major	卅	卅	P. miso	卅	卅	N 15	卅	卅
Z. soya	++	+	N 3	卅	卅	N 24	卅	卅
Z. salsus	卅	卅	N 5	卅	++	N 28	卅	卅
Z; miso α	卅	++	A 6	卅	卅	A 31	卅	卅
S. miso α	卅	卅	N 8	卅	卅	A 34	卅	卅

nitrogen under this condition because of the good growth with ammonium lactate in the other saline medium. In this experiment, the initial pH was 5.0 in the NaCl-free media and 4.4~4.6 in the saline media, being independent of the kind of nitrogen sources, and the final pH was 2.8~3.0 in the ammonium sulfate medium of both plain and saline states which was much lower than 3.4~3.8 in the media of ammonium lactate and casein hydrolyzate. Therefore, it was considered that in the saline medium, inhibition of the growth might be caused by the lowering of pH below 3.0. This idea was confirmed by the fact that if potassium citrate-citric acid buffer was initially added to the above ammonium sulfate medium (100 g of potassium citrate and 25 g of citric acid per 1 l, and 25 ml of this buffer solution was added to 1 l of the medium), sufficient growth was now observed even in the saline medium of 18% NaCl. In this case, initial pH of the medium was 5.0 but the final pH remained at 3.6~3.8. (Table II and Fig. 2)
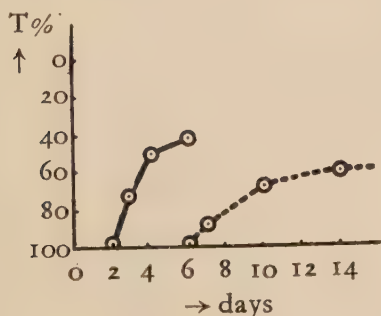
From these results, it is quite clear that the growth

FIG. 2. Growth Curve of Z. major in the Ammonium sulfate Medium Added with the Buffer.
O— —O: NaCl 0%   O---O: NaCl 18 %

of osmophilic yeasts in the environment of high concentration of sodium chloride depends closely upon the pH value of the medium. This will be later investigated in detail.

b) Assimilation of α-Alanine, Glutamic acid and Aspartic acid. As the osmophilic yeasts were able to utilize ammonium-N as the sole sourse of nitrogen even in the saline environment of 18% NaCl provided the pH of the media was suitable, it was expected that in such case, they might utilize, α-alanine, glutamic acid and aspartic acid which had been known as the gateway to organic nitrogen from inorganic nitrogen. The results of the experiment proved the above expectation. (Table III)

Media: As the sole source of nitrogen, each of the following α-alanine, glutamic acid and aspartic acid was added to the basal medium in the amount of 0.03% as the total nitrogen. The basal medium and the methods of the experiment are the same as those shown in Table I.

(2) Effects of Vitamins on the Growth of Osmophilic Yeasts in a Medium of a High Concentration of Sodium Chloride. Burkholder[8] made a comprehensive study on the vitamin requirement of yeasts of many genera and species, having classified them into types based on their requirement. Lochhead and Landerkin[9] also investigated the vitamin requirement of 23 strains of sugar-tolerant yeasts in the glucose 40% medium and classified them into 3 groups on the basis of their requirement of Ca-pantothenate: all of the tested yeasts required biotin, and for some of them, inositol and thiamin were stimulative. English[10] reported that a sugar-tolerant yeast belonging to *Sacch. rouxii* required biotin only.

8) P.R. Burkholder, I. McVeigh and D. Moyer, *J. Bact.* **48**, 385 (1944).
9) A.G. Lochhead and G.B. Landerkin, *J. Bact.* **44**, 343 (1942).
10) M.P. English, *J. Gen. Microbiol.* **10**, 328 (1954).

TABLE III
ASSIMILATION OF α-ALANINE, GLUTAMIC ACID OR ASPARTIC ACID BY THE OSMOPHILIC YEASTS IN THE PLAIN AND THE SALINE NEDIA

Strains	α-alanine		glutamic acid		aspartic acid	
	NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%
Z. major	++	++	++	++	++	++
Z. soya	+	+	+	+	+	+
Z. salsus	++	++	++	++	++	++
Z. miso α	++	++	+++	+++	+++	+++
S. miso α	++	++	+++	+++	+++	+++
P. miso	+++	+++	+++	+++	+++	+++
N 5	++	+	++	++	++	++
N 8	++	++	+++	+++	+++	+++
N 24	++	++	+++	+++	+++	+++
A 31	++	++	+++	+++	+++	+++
A 34	+++	++	+++	++	+++	++

TABLE IV
VITAMIN REQUIREMENT OF OSMOPHILIC YEASTS IN THE PLAIN AND THE SALINE MEDIUM

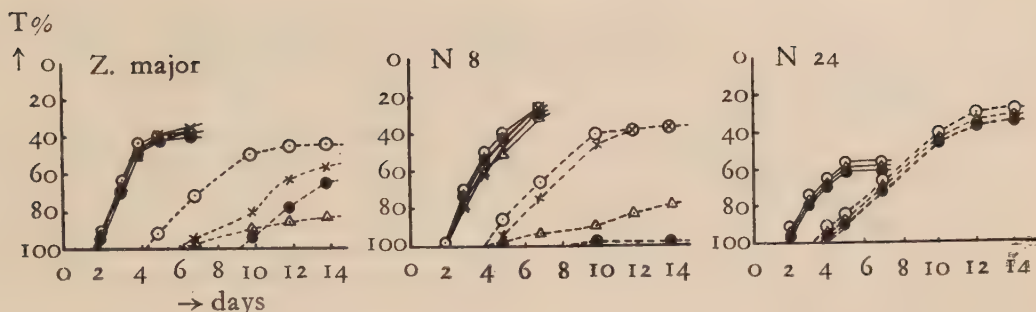| Strains	NaCl % of the medium	no vitamin	all eight vitamins	All the eight vitamins minus							
				biotin	Ca-pantothenate	thiamin	inositol	niacin	pyridoxine	p-amino benzoic acid	riboflavin
Z. major	0%	−	+++	−	++	+++	+++	+++	+++	+++	+++
	18%	−	+++	−	++	+++	+++	+++	+++	+++	+++
Z. soya	0%	−	++	−	++	++	++	++	++	++	++
	18%	−	+	−	±	+	+	+	+	+	+
Z. salsus	0%	−	+++	−	++	+++	+++	+++	+++	+++	+++
	18%	−	+++	−	++	+++	+++	+++	+++	+++	+++
Z. miso α	0%	−	+++	−	++	+++	+++	+++	+++	+++	+++
	18%	−	++	−	++	++	+	++	++	+++	+++
S. miso α	0%	−	+++	−	++	+++	+++	+++	+++	+++	+++
	18%	−	+++	−	±	+++	±	+++	+++	+++	+++
P. miso	0%	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	18%	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
N 3	0%	−	+++	−	++	+++	+++	+++	+++	+++	+++
	18%	−	+++	−	±	+++	+++	+++	+++	+++	++
N 5	0%	−	+++	−	++	+++	+++	+++	+++	+++	+++
	18%	−	+++	−	++	++	+	++	++	++	++
A 6	0%	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	18%	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
N 8	0%	−	+++	−	+++	+++	+++	+++	+++	+++	+++
	18%	−	++	−	±	++	+	+++	+++	+++	+++
N 15	0%	−	++	±	+++	+++	+++	+++	+++	+++	+++
	18%	−	++	−	+++	++	+++	+++	+++	+++	+++
N 24	0%	−	+++	±	+++	−	+++	+++	+++	+++	+++
	18%	−	+++	−	+++	+++	+++	+++	+++	+++	+++
N 28	0%	−	+++	−	+++	+++	+++	+++	+++	+++	+++
	18%	−	+++	−	++	+++	+	+++	+++	+++	+++
A 31	0%	−	++	−	++	+++	+++	++	+++	+++	+++
	18%	−	++	−	++	+++	+++	++	+++	+++	+++
A 34	0%	−	+++	−	+++	+++	+++	+++	+++	+++	+++
	18%	−	+++	−	−	++	+++	++	++	+++	+++

FIG. 3. Growth Curve of the Osmophilic Yeasts in each Vitamin Dificient Media with or without Sodium Chloride.

Solid line: NaCl 0%,   Broken line: NaCl 18%,
○: Presence of all vitamins,   ×: Thiamin omitted,
△: Inositol omitted,   ●: Ca-pantothenate omitted

It was also shown by Sato and Uemura[11] that *Z. major* distinctly required more inositol for its growth in the NaCl 10% medium than in the NaCl-free medium. Comparative studies on vitamin requirements in both the plain and the saline medium by the various salt-tolerant yeasts were carried out. (Table IV and Fig. 3)

Media: Ten kinds of media shown in Table IV were used. In these media, ammonium sulfate was used as a nitrogen source and thereto potassium citrate—citric acid buffer was added and each vitamin was omitted one by one.

The methods are the same as those employed in (1).

In both the plain and saline media, two strains of *Pichia* sp. (*Pichia miso* and A 6) could grow without the presence any vitamin but all the other strains essentially required biotin. For one strain (N 24),

thiamin as well as biotin was essential. Pyridoxine, niacin, *p*-amino benzoic acid and riboflavin were not required for all strains regardless to NaCl concentrations of the media. The requirement of Ca-pantothenate, thiamin and inositol was different according to the strains and the NaCl concentrations of the media. Inositol and thiamin were more or less stimulative in the saline medium for the yeasts which did not require these vitamins in the plain media. Ca-pantothenate was somewhat stimulative in the plain medium for the yeasts except *Pichia* sp. and N 24, and its requirement was much more exact in the saline medium.

(3) Effects of Organic Bases, Yeast Nucleic Acid and Glutathion on the Growth of Osmophilic Yeasts in the Medium of a High Concentration of Sodium Chloride. Since purine and pyrimidine bases are essential building blocks for the

TABLE V

EFFECTS OF PURINE AND PYRIMIDINE BASES, RIBONUCLEIC ACID AND GLUTATHION ON THE GROWTH OF OSMOPHILIC YEASTS IN BOTH THE PLAIN AND SALINE MEDIA

Strains	Basal medium only		With organic bases		With ribonucleic acid		With glutathion	
	NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%
Z. major	₩	₩	₩	₩	₩	₩	₩	₩
A 6	₩	₩	₩	₩	₩	₩	₩	₩
N 8	₩	₩	₩	₩	₩	₩	₩	₩
N 24	₩	₩	₩	₩	₩	₩	₩	₩
A 31	₩	₩	₩	₩	₩	₩	₩	₩
A 34	₩	₩	₩	₩	₩	₩	₩	₩

11) M. Sato and T. Uemura, Lecture read at the meeting of the Agr. Chem. Soc. of Japan (March 31, 1955).

formation of nucleic acid, and glutathion is regarded as the antisalinity factor for *Escherichia coli* by Matsu-
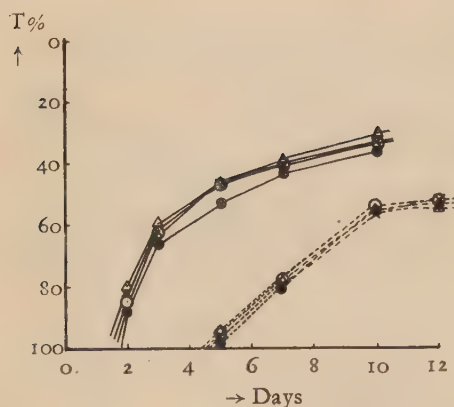
FIG. 4. Growth Curve of *Z. major* in Media added with Organic Bases, Ribonucleic Acid or Glutathion.

○: Basal medium,   ●: With organic bases,
△: With ribonucleic acid,   ×: With glutathion,
Solid line: NaCl 0%,   Broken line: NaCl 18%

yama[12], effects of them on the growth of the osmophilic yeasts in the medium of a high concentration of sodium chloride were tested. The results obtained did not show any stimulative effects. (Table V and Fig. 4)

Media: (1) A basal medium using ammonium sulfate as a nitrogen source and buffered with potassium citrate- citric acid mixture. (2) (1)+NaCl 18%. (3) (1)+purine bases (adenine, guanine and xanthin are added each 10 mg per 1 l of the medium) and

pyrimidine base (uracil 10 mg per l). (4) (3)+NaCl 18%. (5) (1)+ribonucleic acid 0.25%. (6) (5)+NaCl 18%. (7) (1)+glutathion 10^{-3} mol per l. (8) (7)+NaCl 18%.

(4) Influence of the Initial pH of the Medium on the Growth of the Osmophilic Yeasts in the Condition of a High Sodium Chloride Concentration. As shown in experiment (1), in the medium containing ammonium sulfate as a N-source and NaCl in a high concentration as 18%, the growth of the osmophilic yeasts, was found to be extremely poor owing to the rapid decrease of the pH just in the beginning of growth, but this poisonous effect could easily be avoided by buffering the medium at an appropriate pH. Then, further investigations are desirable to be carried out concerning the influences of pH on growth in the environment of a high concentration of sodium chloride.

Sherman and Holm[13], from the pH-growth rate curve of *Escherichia coli* and *Alcaligenes faecalis*, observed that a concentration of 0.2 mol was optimal and caused a large degree of broadening of the pH range for their rapid growth compared with no addition of sodium chloride to 1% peptone medium. Matsuyama[12] found that the effect of pH of the medium on the growth of *Escherichia coli* agreed with the observation made by Sherman and Holm[13] in 0.1~0.3 mol of NaCl, but with a sublethal concentration of NaCl as 1 mol, its growth was limited
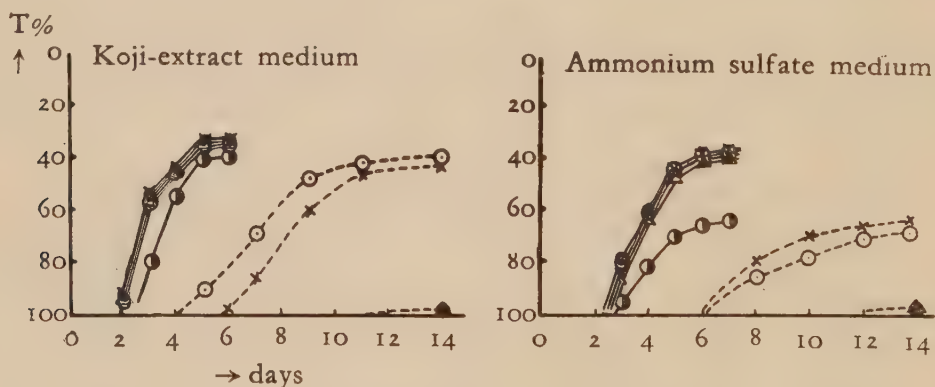

FIG. 5. Growth Curve of *Z. major* in the Plain and Saline Medium at Various pH Values.

◑: pH 3.0,   ○: pH 4.0,   ×: pH 5.0,
●: pH 5.8,   △: pH 6.6,
Solid line: NaCl 0%,   Broken line: NaCl 18%,

12) A. Matsuyama, *J. Agr. Chem. Soc. Japan*, **28**, 299, 304 (1954).

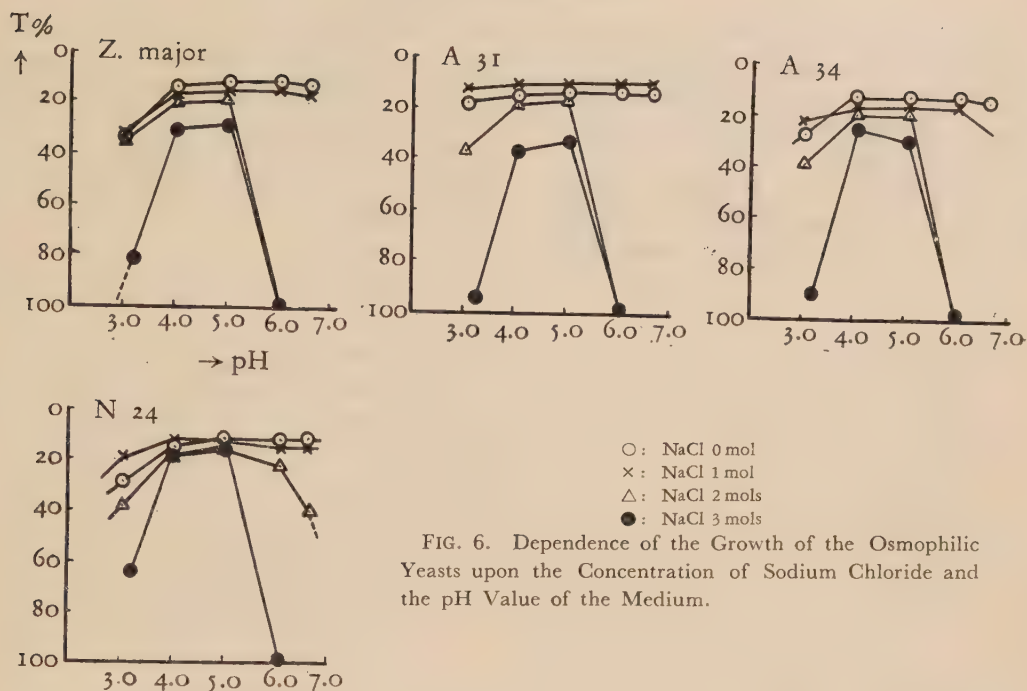13) J.M. Sherman and G.E. Holm, *J. Bact.* **7**, 465 (1922)

FIG. 6. Dependence of the Growth of the Osmophilic Yeasts upon the Concentration of Sodium Chloride and the pH Value of the Medium.

○ : NaCl 0 mol
× : NaCl 1 mol
△ : NaCl 2 mols
● : NaCl 3 mols

within the range of pH 6~7. By Joslyne and Cruess[14], it was observed that the lower the pH of the medium, the lower the concentration of salt required to inhibit the growth of *Mycoderma*, the film-yeast in salted foods, became.

(a) **The Growth of *Z. major* in the Plain and Saline Medium at Various pH Values.** *Media :* The pH of the medium was aseptically adjusted with HCl and NaOH. (1) Koji extract medium. (2) Basal medium containing ammonium sulfate as a nitrogen source and McIlvain buffer of different pH values.

As shown in Fig. 5, in the plain medium the growth of *Z. major* was almost equal in the range of pH 3~7 without regard to the kind of the media used, but in the saline medium their growth was limited within the range of pH 4~5.

(b) **The Growth of Osmophilic Yeasts in the Medium of Various Concentrations of Sodium Chloride and Hydrogen Ion.** *Media :* Koji extract, the pH being adjusted aseptically with HCl and NaOH.

The results in Fig. 6 show that the growth of

14) M.A. Joslyne and W.V. Cruess, *Hilgardia*, **4**, 201 (1929).

yeasts was uniformly good in the range of pH 3.0~6.6 in a concentration of 1 mol of sodium chloride, but the growth was limited in the range of pH 4.0~5.0 in the media of more than 2 mols of sodium chloride.

DISCUSSION

Since the osmophilic yeasts were able to utilize ammonium-N as the sole source of nitrogen in the medium of a high concentration of sodium chloride, it is presumable that the synthesizing process of cellular protein necessary for growth from ammonium-N may proceed sufficiently even in the environment of a hgih concentration of sodium chloride.

English[10] has stated that a sugar-tolerant yeast of the *Saccharomyces rouxii* type was able to grow in such a wide pH range as 1.8~8.0 in a high glucose concentration of 46%. On the contrary, in the medium of a high concentration of sodium chloride, the growth of the osmophilic yeasts was limited within pH 4.0~5.0 as shown in Figs. 5 and

6. The different nature of the salt-tolerance and sugar-tolerance of the osmophilic yeasts was also shown in respect of this pH dependence in addition to the results described in the previous paper.

Studies on Osmophilic Yeasts

Part III.   Classification of Osmophilic Soy and Miso Yeasts

By Hiroshi Ōnishi

Noda Institute for Scientific Research

Most of the salt-tolerant yeasts isolated from soy-sauce and miso-paste belonged to *Saccharomyces rouxii* Boutroux.   Some of them produced luxuriant pellicles on the medium containing sodium chloride, though pellicles were not formed on the medium without salts. These strains were separated from the species, in a variety, *Saccharomyces rouxii* Boutroux var. *halomembranis* nov. var.   A few strains were identified as *Saccharomyces acidifaciens* (Nickerson) Lodder et Kreger-van Rij.   Still further, two new species, *Torulopsis halophilus* nov. sp. and *Torulopsis nodaensis* nov. sp. were also described.   These two species showed high salt-tolerance, especially the former was facultative halophile.   Both species were able to assimilate nitrate but not to form pellicles, and differed from each other in the fermentation and assimilation of sugars.

INTRODUCTION

As for the osmophilic yeasts, many new species and varieties have been described[1][2]. Most of them belong to *Zygosaccharomyces* and some others to *Debaryomyces*, *Pichia*, *Torulopsis* and *Saccharomyces* etc.   It seems that they generally exist in haplophase in an environment of a high concentration of sugars or salts.   In the monogragh on the taxonomy of yeasts by Lodder and Kreger-van Rij[3], subgenera *Zygosaccharomyces* is no longer recognized, on the basis of the fact that no fundamental difference exists between the types of sexual reproduction shown by the species of *Saccharomyces* and *Zygosaccharomyces*.   In this classification, almost all of the *Zygosaccharomyces* known as the osmophilic yeasts, were included into only two species and one variety, *Saccharomyces rouxii* Boutroux, *Saccharomyces rouxii* Boutroux var. *polymorphus* Lodder et Kreger-van Rij and *Saccharomyces*

mellis (Fabian et Quinet) Lodder et Kreger-van Rij.   For instance, *Zygosaccharomyces major*[4], *Z. soya*[5], *Z. salsus*[4] and *Z. japonicus*[6] known as soy yeasts, were all included in one species, *Saccharomyces rouxii*.

This paper is concerned with a taxonomic study on the yeast strains which play an important role in soy-brewing, isolated from soy-mashes by the author and the collected strains labelled as *Z. major*, *Z. soya*, *Z. salsus*, *Z. japonicus*, *Z. miso*[7] and *Sacch. miso*[7].

METHODS

The methods employed and the classification system referred here were essentially the same as those outlined in the monograph by Lodder and Kreger-van Rij[3], though certain modifications and additions were made in connection with the taxonomic test carried out by the Dutch workers.

The outlines of the examination were as follows:

1)   A.T. Henrici, *Bact. Rev.* **5**, 97 (1941).

2)   E.M. Mrak and H.J. Phaff, *Ann. Rev. Microbiol.* **2**, 1 (1948).

3)   J. Lodder and N J.W. Kreger-van Rij, The Yeasts, a taxonomic study (1952).

4)   T. Takahashi and M. Yukawa, *J. Coll. Agr. Tokyo Imp. Univ.* **5**, 227 (1915).

5)   K Saito, *Centr. Bakt. Parasitenk.* **Abt. II. 17**, 20, 101, 152 (1907).

6)   K. Saito, *Botan. Magazine Tokyo*, **23**, 96 (1909).

7)   M. Mogi, *J. Agr. Chem. Soc. Japan*, **12**, 367 (1936), **14**, 951 (1938); **15**, 921, 1023, 1221 (1939); **16**, 7 (1940); **18**, 543, 733, 940 (1942).

TABLE I MORPHOLOGICAL, CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE TESTED YEASTS

Species allocation	*Saccharomyces rouxii* Boutroux	*Saccharomyces rouxii* Boutroux var. *halomembranis* nov. var.	*Saccharomyces acidifaciens* (Nickerson) Lodder et Kreger-van Rij	*Saccharomyces acidifaciens* (Nickerson) Lodder et Kreger-van Rij var. *halomembranis* nov. var.	*Torulopsis halophilus* nov. sp.	*Torulopsis nolaensis* nov. sp.
Vegetative cells (after 6 days' cultivation in koji extract, at 30°)	Cells are round or short oval, single or in pairs. The cells usually measure $(5\sim8)\times(5\sim10)$ μ.	Same as *Saccharomyces rouxii*	Cells are round or oval, single or in pairs. The cells usually measure $(4\sim6)\times(7\sim10)$ μ.	Same as *Saccharomyces acidifaciens*	Cells are oval or short ellipsoidal, single or in pairs. The cells usually measure $(2\sim4)\times(3\sim5)$ μ.	Cells are oval, single or in pairs. Cells usually measure $(2\sim4)\times(3\sim5)$ μ.
Growth on koji agar, after 2 weeks, at 30°	The streak culture is light cream colored, dull glistening, raised and almost smooth.	The streak culture is light cream colored, dull, raised and wrinkled.	The streak culture is cream-colored, dull glistening, raised and smooth.	The streak culture is cream-colored, dull, raised and slightly wrinkled.	The streak culture is white grey, glistening, flat and smooth.	The streak culture is greyish brown, almost flat, dull shining and almost smooth.
Slide culture on potato agar	No pseudomycelium	No pseudomycelium	No pseudomycelium	No pseudomycelium	No pseudomycelium	No pseudomycelium
Ascospore formation	Isogamous or heterogamous conjugation may precede ascus formation. 1~4 spores are found per ascus. Spores are round.	Isogamous or heterogamous conjugation may precede ascus formation. 1~4 spores are found per ascus. Spores are round.	Isogamous or heterogamous conjugation may precede ascus formation. 1~4 spores are found per ascus. Spores are round.		Not found on three sporulation media observed over a three months-period.	Not found on three sporulation media observed over a three months-period.
Growth in koji extract { NaCl-free	Ring—no film	Cream-like island or shred of film are formed.	Ring—no pellicle	Cream-like island or shred of film are formed.	Ring—no pellicle	Ring—no pellicle
NaCl 18%	Good growth Ring—no film	Good growth and a thick wrinkled film is formed.	Good growth. Ring—no pellicle	Good growth and a thick wrinkled pellicle is formed.	Good growth. Ring—no pellicle	Good growth. Ring—no pellicle
Fermentation of sugars { Fermented	Glucose and maltose	Glucose and maltose	Glucose	Glucose	Glucose	Glucose and galactose
Unfermented	Galactose, saccharose and lactose	Galactose, saccharose and lactose	Galactose, maltose, saccharose and lactose	Galactose, maltose, saccharose and lactose	Galactose, maltose, saccharose and lactose	Maltose, saccharose and lactose
Assimilation of sugars { Assimilated	Glucose, galactose and maltose. Saccharose (variable)	Glucose, galactose and maltose. Saccharose (variable)	Glucose and Galactose.	Glucose and Galactose	Glucose, galactose, saccharose and maltose	Glucose, galactose and saccharose
Not assimilated	Lactose	Lactose	Maltose, saccharose and lactose	Maltose, saccharose and lactose	Lactose	Maltose and lactose
Assimilation of nitrate	Nagative	Negative	Negative	Negative	Positive	Positive
Growth in ethanol	No growth	No growth	No growth	No growth	Growth	Good growth
Splitting of arbutin	Negative	Negative	Negative	Negative	Negative	Negative
Outstanding characteristics					Facultative halophilic. Besides biotin, thiamin is essentially required.	Besides biotin, thiamin is essentially required.

(1) Characteristics of vegetative reproduction.

The formation of pseudomycelium was observed in a slide culture with potato agar.

(2) Shape and size of the cells.

(3) Ascospore formation.

Three media, Gorodokowa agar, V–8 agar[8] and diluted soy-sauce (four times) were used. The final observation was made after three months' cultivation.

(4) Macromorphological characteristics of the cultures on koji agar.

(5) Pellicle formation.

It was observed in koji extract with and without sodium chloride, salt-tolerance of the tested yeasts being examined at the same time.

(6) Fermentation of sugars.

The fermentation of 2% sugar solution in yeast extract was tested in a Durham tube and an Einhorn tube. The sugar were: glucose, galactose, maltose, saccharose and lactose. The final reading was made after 10 days.

(7) Assimilation of sugars.

Bacto yeast-nitrogen base was employed as the basal liquid medium.

(8) Assimilation of nitrate was tested with a Bacto yeast-carbon base.

(9) Ethanol as a sole source of carbon.

(10) Splitting of arbutin.

EXPERIMENTAL AND RESULTS

First of all, the salt-tolerance of the tested yeasts was examined and it was confirmed that the strains classified below, were all able to grow in a 18% NaCl medium.

The 87 tested strains were classified as follows:

(1) *Saccharomyces rouxii* Boutroux

(2) *Saccharomyces rouxii* Boutroux var. *halomembranis* nov. var.

(3) *Saccharomyces acidifaciens* (Nickerson) Lodder et Kreger-van Rij.

(4) *Saccharomyces acidifaciens* (Nickerson) Lodder et Kreger-van Rij var. *halomembranis* nov. var.

(5) *Torulopsis halophilus* nov. sp.

(6) *Torulopsis nodaensis* nov. sp.

Their morphological, cultural and physiological characteristics are summarized in Table I.

DISCUSSION

Of the 87 tested strains, 78 strains belonged to *Saccharomyces rouxii* Boutroux, and among them 24 strains produced thick wrinkled pellicles on the medium containing sodium chloride, though pellicle formation was absent in the liquid media without salts. This characteristic has previously been recognized by Takahashi and Yukawa[4] on *Z. salsus*, the film yeast of soy, and also by Mogi[7] on some strains of miso yeasts. Recently, Etchells and Bell[9] described *Zygosaccharomyces halomembranis* which was isolated from films on commercial cucumber brines and showed the same characteristic of pellicle formation as mentioned above. This species is presumed to be synonymy with *Sacch. rouxii* for it ferments glucose and maltose, and not galactose, saccharose and lactose. They are, of course, clearly distinguished from *Pichia* and *Hansenula* in the point of lacking early development of dry matte pellicles on ordinary media without salts. However, the property of luxuriant pellicle formation on the media containing NaCl, e.g. soy-sauce, is considered not only to be a very obvious characteristic for the taxonomy of yeasts but also a very important one pertaining to the industrial and applied standpoints, e.g. the preservation of soy-sauce. The author, therefore, is of opinion that these strains should be separated from the species, *Sacch. rouxii* Boutroux, in a variety, *Sacch. rouxii* Boutroux var. *halomembranis* nov. var.

It was noticeable that a half of the collected strains labelled as *Z. salsus* and *Z. japonicus*, could not form pellicles on the medium containing NaCl, though it was not apparent whether their ability of pellicle formation was lost or not during the stock culture for many years, yet, however they agreed with *Sacch. rouxii* in all other properties.

Pellicle formation on the media of NaCl-free and 6~9% NaCl by many species of *Saccharomyces* and *Zygosaccharomyces* received from the Nagao Institute in Tokyo, Japan, and by the strains placed in *Sacch. rouxii*

8) L. J. Wickerham, M.H. Flickinger and K.A. Burton, *J. Bact.* **52**, 611 (1946).

9) J.L. Etchells and T.A. Bell, *Food Technol.* **4**, 77 (1950).

TABLE II

SALT-TOLERANCE AND SUGAR-TOLERNCE OF VARIOUS STRAINS OF *SACCH. ROUXII*.

Strains	NaCl					Glucose		Saccharose	
	0%	10%	12.5%	15%	18%	80%	90%	80%	90%
Zygosacch. amoeboideus	++	++	+	+	—	+	—	+	+
Z. citrus	++	++	++	++	++	+	—	+	+
Z. felsineus	++	++	++	++	—	+	—	+	+
Z. gracilis	++	++	++	++	—	+	—	+	+
Z. gracilis var. italicus	++	++	++	++	±	+	—	+	+
Z. mellis	++	++	++	++	±	+	—	+	+
Z. nussbaumeri	++	++	—	—	—	+	—	+	+
Z. polymorphus	++	+	+	—	—	+	—	+	+
Z. rugosus	++	++	++	++	—	+	—	+	+
Z. variabilis	++	++	++	—	—	+	—	+	+
N 5*	++	++	++	++	++	+	—	+	+
N 8*	++	++	++	++	++	+	—	+	+
A 34*	++	++	++	++	++	+	—	+	+
Torulopsis halophilus	++	++	++	++	++	+	—	+	+
T. nodaensis	++	++	++	++	++	+	—	+	+

* These strains were isolated from soy-mashes by the author and then placed in *Sacch. rouxii*.

supplied from Centraalbureau voor Schimmelcultures, Delft, Holland was observed. The strains tested were as follows:

Sacch. anamensis, S. awamori, S. bayanus, S. cartinoginosus, S. chevalieri, S. dairensis, S. exiguus, S. fragilis, S. intermedius, S. lindneri, S. logos, S. marxianus, S. monacensis, S. mongolicus, S. paradoxus, S. pasteurianus, S. unisporus, S. validus, S. willianus, Zygosacch. bisporus, Z. dairensis, Z. fermentati, Z. mellis acidi, Z. naniwaensis, Z. vini, Z. amoeboideus, Z. citrus, Z. felsineus, Z. gracilis, Z. gracilis var. italicus, Z. mellis, Z. nussbaumeri, Z. polymorphus, Z. rugosus, Z. variabilis.

Of the tested strains, only two, *Z. mellis acidi* and *Z. nussbaumeri*, were able to form prominent pellicles, only on the saline medium. Therefore, it is estimated that few strains with the exception of osmophilic yeasts show such property.

Concerning the fermentability of saccharose by *Sacch. rouxii*, it was shown by Terui and Sase[10] that the utilization of saccharose is detectable, only when saccharose adaptation is permitted under the condition applied, that is to say, a negative result is obtained with the Lodder medium, but on fortification with 0.2% of L-aspartic acid and 1 μg per ml of the biotin, the result becomes positive. The author's observation on the fermentation of saccharose by the strains precultured on koji agar, was in good agreement with the description of Lodder and Kreger-van Rij though some strains showed extremely poor saccharose fermentation after 15~20 days' incubation.

The salt-tolerance of 10 strains of *Sacch. rouxii* received from Centraalbureau voor Schimmelculture, Delft, Holland, was lower than that of soy and miso yeasts, in spite of their same level of sugar-tolerance. (Table II) It is suggested that the salt-tolerance and sugar-tolerance of *Sacch. rouxii* differ considerably, strain by strain, according to their origin.

Spore formation of the 78 strains that belonged to *Sacch. rouxii* and its variety was very difficult, being observable in only two strains. Lodder and Kreger-van Rij have also recognized the difficulty of this

10) G. Terui and M. Sase, *Technol. Rep., Osaka Univ.*, 5, 229 (1955).

species and stated that it might be due to its strong tendency to live in haplophase and to easily lose its ability to form ascospores.

Four strains were identified as *Sacch. acidifaciens* (Nickerson) Lodder et Kreger-van Rij due to the facts that they fermented glucose only, their cell size being relatively large (6~9μ), and the streak culture on koji agar being smooth. Two strains among them, which formed prominent pellicles only on the medium containing NaCl were separated from
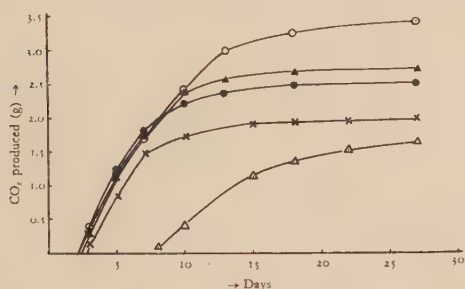

FIG. 1. Fermentation Curve of *Sacch. rouxii* in Media of High Concentrations of NaCl and Sugars.

    ○: the plain medium
    ×: the 10% NaCl medium
    △: the 18% NaCl medium
    ●: the 50% glucose medium
    ▲: the 50% saccharose medium
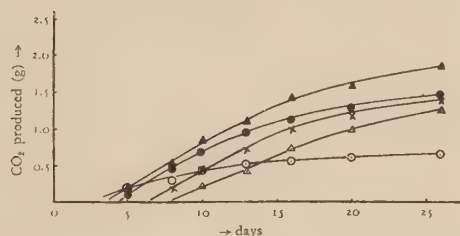

FIG. 2. Fermentation Curve of *Torulopsis halophilus* in the Media of High Concentrations of NaCl and Sugars.

    ○: the plain medium
    ×: the 10% NaCl medium
    △: the 18% NaC. medium
    ●: the 50% glucose medium
    ▲: the 50% saccharose medium

the species in a variety, *Sacch. acidifaciens* var. *holomembranis* nov. var.

Two strains isolated from soy mashes in

Noda district by the author, belonged to genus *Torulopsis*, due to the characteristics that are shown in Table I. It is an interesting fact that though both are able to assimilate nitrate very well, neither is capable of forming pellicles on any liquid media with or without NaCl. Furthermore, they required biotin and thiamin essentially[11]. They were different from each other in both the fermentability and assimilability of sugars. As shown in Figs. 1 and 2, one of them showed a better fermentation in the medium of high concentrations of NaCl or sugars than in the ordinary plain medium, unlike the fermentation curve shown by *Sacch. rouxii*, that is to say, it is facultative halophilic.

This halophilic yeast, in many taxonomic properties, resembles *Torulopsis etchellsii* Lodder et Kreger-van Rij which was isolated from cucumber pickles and named *Brettanomyces sphericus* by Etchells and Bell[12], and thereafter renamed as above by Lodder and Kreger-van Rij, but that is distinguished from *T. etchellsii* in respect of its fermentability and assimilability of sugars, and utilization of ethanol. It also differs from *Hansenula minuta*[13] in its assimilability of sugars, splitting of arbutin and halophilic property, even when it is sporulated. Accordingly, the author regards it as a new species and has given it the name *Torulopsis halophilus* nov. sp.

The other strain has close resemblance to *Torulopsis halophilus* but differs in its fermentability of galactose and salt-tolerance (non-halophilic). And also, it differs from *Hansenula silvicola* in its assimilation of sugars, pseudomycel formation and utilization of ethanol besides sporulation. It, therefore, is named *Torulopsis nodaensis* nov. sp.

11) H. Onishi, This Bulletin in press.
12) J.L. Etchells and T.A. Bell, *Farlowia*, **4**, 87 (1950).
13) L.J Wickerham, *Tech. Bull.* No. 1029, U.S. Dep. Agric. Washington, D.C., May (1951).

Isolation and Identification of α- and β-Amyrin from the Bark of *Ilex latifolia* Thunberg*

By Kazuyoshi Yagishita

*Department of Chemistry, Kumamoto Women's University***

Two triterpenoid alcohols, i.e., α- and β-amyrin have been isolated from the unsaponifiable fraction of the ethereal extract of *Ilex latifolia* Thunberg (*Aquifoliaceae*). The ratio of the amyrin contents, i.e., α-amyrin to β-amyrin was about 4 : 1. Stearic acid has also been isolated from the fatty-acid fraction of the hydrolysates.

Ilex latifolia Thunberg*** (Japanese name: Tarayo; *Aqufoliaceae*) is an evergreen arbor indigenous to temperate districts of Japan. It is a well-known fact that the bark of this plant contains a certain amount of resinous matter, which is said to be one of the sources of commercial "blue bird-lime".

From the leaves of this plant, ursolic acid was isolated by T. Kariyone et al[1]., and T. Takemoto et al[2]. Later, it was also isolated from the fruits of the same plant by T. Koyama et al[3].

To date, to our knowledge, nothing has yet been reported as having been isolated from the bird-lime of the bark of *Ilex latifolia* Thunberg.

The present paper deals with the isolation and identification of α- and β-amyrin from the unsaponifiable fraction of the ethereal extract of the bark of this plant.

To achieve satisfactory separation of tri-terpenoids, the acetylated mixture of the unsaponifiable fraction was subjected to fractional crystallization using ethanol as the solvent.

From the fraction less soluble in ethanol, there was isolated α-amyrin acetate, which was further deacetylated to yield α-amyrin. From the fraction almost freed of α-amyrin acetate, there was isolated β-amyrin acetate, which was similarly deacetylated to yield β-amyrin.

The ratio of the amyrin contents, i.e., α-amyrin to β-amyrin was about 4 : 1.

An attempt to isolate triterpenoid acid from the ethereal extract of this bark was unsuccessful.

Besides these, stearic acid was found to be present in the fatty-acid fraction of the hydrolysates.

It is of interest to note that β-amyrin has been shown to be a common constituent in all sorts of bird-lime hitherto examined[4] in this laboratory.

EXPERIMENTAL*

Preparation and Hydrolysis of the Resin (Bird-lime). The bark of *Ilex latifolia* Thunberg (1.53 kg),

* Part IV: This Bulletin, **21**, 77 (1957). This is Part V of a series of papers entitled as "Studies of Constituents of Various Sorts of Bird-lime"; Presented at the General Meeting, West Japan Branch of the Agricultural Chemical Society of Japan, held at Kumamoto Women's University, Kumamoto City, Nov. 10, 1956.

** Ooemachi, Kumamoto City, Japan.

*** T. Makino, K. Nemoto, "*Flora of Japan*", 2nd ed., 672 (1931).

1) T. Kariyone, Y. Hashimoto, T. Kikuchi, *J. Pharm. Soc. Japan*, **69**, 314 (1949).

2) T. Takemoto, K. Takahashi, *Yakugaku Kenkyu*, **22**, 301 (1952).

3) T. Koyama, I. Kato, *Kumamoto Pharm. Bull.* (Japan), No. 1, 41 (1951).

4) a) S. Iseda, *J. Pharm. Soc. Japan*, **72**, 1611 (1952); b) S. Iseda, K. Yagishita, N. Toya, ibid., **74**, 422 (1954); c) K. Yagishita, This Bulletin, **20**, 97; 106 (1956); d) K. Yagishita, ibid., **21**, 77 (1957).

* All melting points were corrected; Rotations were measured in CHCl₃ solution, using 1-dm tubes.

collected in Minamata City, Kumamoto Prefecture in the spring of 1956, was cut into chops, air-dried, and finally dried at 80° for 8 hrs.. The yield was 1.16 kg (75.81%).

After exhaustion with ether of the dried material for 56 hrs., there was obtained 222 g (19.12%) of the dried bark of the tan-colored extract soluble in 5–6 times its weight of cold benzene. After being refluxed with 10% benzene-ethanolic KOH-solution (benzene-ethanol 1:1) for 50 hrs., the reaction mixture was filtered while hot. After removal of the solvent *in vacuo*, the residue was diluted with water (2.5 l), and subjected to complete extraction with ether for 40 hrs..

The aqueous layer was combined with the washings, acidified with HCl, and extracted with ether to yield a fatty-acid fraction (1) (89.5 g: 7.71%), whereby no separation of potassium salt of triterpenoid acid was observed between alkaline and ethereal layers.

After drying and removal of ether, there remained a pale yellow crystalline mass (132.0 g: 11.37%), which was refluxed for 1 hr. with 20 times its weight of ethanol, cooled, and then filtered. The filtrate was concentrated to a small volume, and refluxed with an appropriate amount of ethanol as described above. After repetition of the above procedure, there were finally obtained: a) a yellowish syrupy fraction (II) (34.5 g: 2.97%) and b) colorless needles, m.p. 178–187° (III) (97.0 g: 8.36%).

Isolation of α-Amyrin Acetate. To a solution of 97.0 g of III in 250 ml of benzene, was added 110 ml of acetic anhydride, and the mixture was refluxed for 3 hrs.. After removal of the solvents *in vacuo*, the remaining crude acetate was washed with water, dried, and then refluxed with 50 times its weight of ethanol to yield a clear solution, indicative of the absence of triterponoids, such as ilexol[5] or taraxol[6]. After fractional recrystallization from ethanol, there were obtained: a) the less-soluble fraction (IIIa) (89.0 g), m.p. 216–220°, as plates, and b) the more-soluble fraction (IIIb) (8.0 g), m.p. 228–235°, as prismatic needles.

An amount of 89.0 g of IIIa was further recrystallized several times from ethanol to yield plates (33.5 g), which melted at 223.5–224.5° either alone or on admixture with an authentic specimen of α-amyrin

acetate[4a,4b]. $[\alpha]_D^{19}+76.2°$ (c, 0.752).

Anal. Found: C, 81.75; H, 11.33. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

α-Amyrin. Hydrolysis of 2 g of α-amyrin acetate (m.p. 223.5–224.5°) with 3% ethanolic KOH-solution afforded free alcohol (1.8 g), which after three recrystallizations from ethanol, melted at 187° either alone or on admixture with an authentic specimen of α-amyrin[4b]. $[\alpha]_D^{19}+83.1°$ (c, 0.752).

Anal. Found: C, 84.21; H, 11.97. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

α-Amyrin Benzoate. A solution of 0.8 g of α-amyrin (m.p. 187°) in 1 ml of pyridine was benzoylated in the usual manner with 1.5 ml of benzoyl chloride. After several recrystallizations from ethanol of the crude benzoate, there were obtained plates (0.8 g), melting at 194–195°. (Recorded[b] m.p. 194–195°).

Anal. Found: C, 83.56; H, 10.53. Calcd. for $C_{37}H_{54}O_2$: C, 83.72; H, 10.25.

Hydrolysis of 0.2 g of this benzoate with 3% ethanolic KOH-solution afforded α-amyrin, and melted at 187° either alone or on admixture with an authentic specimen[4b].

Isolation of β-Amyrin Acetate. a) An amount of 8.0 g of IIIb was further recrystallized several times from ethanol or ethyl acetate to yield long prismatic needles (3.2 g), m.p. 239–240°. b) An amount of 34.5 g of II was acetylated in the usual manner with benzene-acetic anhydride mixture. Recrystallized several times from ethanol, the crude acetate (12.0 g) afforded prismatic needles (4.8 g), melting at 239–240°. $[\alpha]_D^{19}+83.5°$ (c, 0.684).

Both of these specimens did not depress the melting point on admixture with an authentic specimen of β-amyrin acetate[4c].

Anal. Found: C, 82.10; H, 11.28. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

β-Amyrin. β-Amyrin acetate (0.5 g) was refluxed with 3% ethanolic KOH-solution for 1.5 hrs. to yield free alcohol, which after three recrystallizations from ethanol afforded needles (0.4 g), melting at 196°. $[\alpha]_D^{19}+84.9°$ (c, 0.576).

Anal. Found: C, 84.25; H, 11.99. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

β-Amyrin Benzoate. β-Amyrin (0.2 g) was benzoylated in the usual manner with pyridine and benzoyl chloride. After several recrystallizations from ethanol of crude benzoate, there were obtained plates (0.2 g), melting at 232°. (Recorded[4c] m.p. 232°). $[\alpha]_D^{19}+87.4°$ (c, 0.852).

5) a) S. Iseda, *J. Pharm. Soc. Japan*, **72**, 1064 (1952); b) K. Yagishita, unpublished work. The further occurrence of ilexol has now been demonstrated in "blue bird-lime" from the bark of *Ilex crenata* Thunberg (*Aquifoliaceae*).

6) S. Burrows, J.C.E. Simpson, *J. Chem. Soc.*, **1938**, 368.

Anal. Found: C, 83.57; H, 10.58. Calcd. for $C_{37}H_{54}O_2$: C, 83.72; H, 10.25.

Hydrolysis of 0.1 g of this benzoate with 3% ethanolic KOH-solution afforded β-amyrin, melting at 196° either alone or on admixture with an authentic specimen[4c].

Isolation of Stearic Acid. An amount of 89.5 g of I was dissolved in a small amount of ethanol and left at room temperature for several days. There separated granular crystals, which after several recrystallizations from ethanol afforded scales, melting at 70–71° either alone or on admixture with an authentic specimen of stearic acid[7]. The yield was 13.5 g.

Anal. Found: C, 77.13; H, 12.41. Calcd. for $C_{19}H_{36}O_2$: C, 76.97; H, 12.24.

The amide of this acid (1.0 g) was prepared in the usual manner and recrystallized several times from ethanol to yield lamellar crystals (1.0 g), which melted at 108°, undepressed on admixture with an authentic specimen[7].

7) K. Yagishita, S. Iseda, *J. Agr. Chem. Soc. Japan*, **29**, 964 (1955).

Further Occurrence of Ilexol along with α-and β-Amyrin in the Bird-lime from the Bark of *Ilex crenata* Thunberg*

By Kazuyoshi Yagishita

*Department of Chemistry, Kumamoto Women's University***

Received December 29, 1956

Three triterpenoid alcohols, i.e., ilexol, α- and β-amyrin have now been isolated from the unsaponifiable fraction of the ethereal extract of the bark of *Ilex crenata* Thunberg (Family: *Aqufoliaceae*). Stearic acid has also been isolated from the fatty-fraction of the hydrolysates.

In view of phylogenetic and taxonomic standpoints, the occurrence in various sorts of bird-lime of triterpenoids and β-sitosterol discussed in this paper.

In previous paper[1],[2] of this series, it has been shown that commercial "white bird-lime" and bird-lime from the bark of *Ilex integra* Thunberg, contain a mixture of triterpenoid alcohols capable of being fractionated to yield ilexol, α- and β-amyrin, and lupeol, respectively.

It has also been show that commercial "blue bird-lime" used previously[2], contains a mixture of ilexol, α- and β-amyrin, and β-sitosterol capable of being fractionated into single entities.

Ilex crenata Thunberg*** (Japanese name: Inutsuge; Family; *Aquifoliaceae*) is an evergreen tree indigenous to Saghalien, Kuriles, Hokkaido, Honshyu. Shikoku, Kyushu, Loochoo, southern districts of Korean Peninsula, and Quelpaert Island. It is also well-known that the bark of this plant contains an amount of resinous matter, which is being made use of ensnaring little birds, etc.

As to the triterpenoid constituents, to date, to our knowledge, nothing has yet been reported as have been isolated from the bird-lime of this plant.

Separation of a mixture of triterpenoids obtained from this bird-lime has now satisfactorily been achieved, and the description and discussion of the results of experiments of which are the chief aim of this paper.

A mixture of acetates obtained by acetylating the unsaponifiable fraction of this bird-lime, has now been subjected to fractionation, using ethanol as an appropriate solvent.

As reported previously[1],[2], it has been confirmed that the fraction almost insoluble in ethanol, is consisted mainly of ilexol acetate of $C_{32}H_{54}O_2$, m.p. 290–291°, and $[\alpha]_D^{14} - 32.28°$ (c, 0.557), which is deacetylated to yield ilexol of $C_{30}H_{50}O$, m.p. 205–206°, and $[\alpha]_D^{14} - 29.05°$ (c, 0.860).

Further fractionation of the acetate-fractions freed of ilexol has been carried out and now detailed as follows: deacetylation-benzoylation of these fractions has been found to give a mixture of benzoates of α- and β-amyrin capable of being fractionated by using a mixture of ethyl acetate and ethanol (1 : 1) as an appropriate solvent mixture.

In a similar way, as reported previously[2], it has been confirmed that the fraction less-soluble in this solvent mixture, affords α-amyrin benzoate, which is debenzoylated to yield α-amyrin, whereas, the remaining one

* Part V: This Bulletin, **21**, 157 (1957). This is Part VI of a series of papers entitled as "Studies of Constituents of Various Sorts of Bird-lime".

** Ooemachi, kumamoto City, Japan.

1) S. Iseda, *J. Pharm. Soc. Japan*, **72**, 1611 (1952).

2) S. Iseda, K. Yagishita, N. Toya, *ibid.*, **74**, 422 (1954).

*** J. Ohwi, "*Flora of Japan*", Shibundo, Tokyo, 732 (1953).

almost freed of α-amyrin benzoate, afforded β-amyrin benzoate, which is debenzoylated to yield β-amyrin.

From the fatty-acid fraction, stearic acid has also been isolated.

Now it follows from the foregoing results of experiment that a mixture of triterpenoids from the unsaponifiable fraction of bird-lime of *Ilex crenata* Thunberg is capable of being fractionated to yield ilexol, α- and β-amyrin respectively.

EXPERIMENTAL*

Preparation and Hydrolysis of the Resin (Bird-lime). The bark (1.95 kg) of *Ilex crenata* Thunberg collected at Mt. Sannotake in Kumamoto Prefecture in the summer of 1956, was cut into small pieces, air-dried, and thoroughly dried at 60°. The yield was 1.25 kg (64%).

After exhaustive extraction with ether of the dried material for 45 hrs., there was obtained 134.5 g of tan-colored extract (10.75% of the dried bark), which was further reextracted with a suitable amount of cold benzene, leaving a small quantity of a benzene-insoluble, tan-colored granular mass (1.5 g: 0.12%), which gave a blue coloration with $FeCl_3$.

After removal of benzene, the remaining purified resin was refluxed for 40 hrs. with benzene-ethanolic KOH-solution (10%).

After filtration while hot and removal of the solvents *in vacuo*, the residue was treated with ether to yield a fatty-acid fraction (I) (64.0 g: 5.11%), and the un-saponifiable fraction of m.p. 178–187° (II) (68.5 g: 5.46%). Fraction II was refluxed with a suitable amount of ethanol for 1 hr., cooled, and filtered, after which the resulting filtrate was concentrated to a small volume, and refluxed with a suitable amount of ethanol. After repetition of the above procedure, there were finally obtained a) yellowish syrup more-soluble in ethanol (III) (47.5 g: 3.80%), and b) color-less needles of m.p. 180–193° less-soluble in ethanol (IV) (21.0 g: 1.68%).

Isolation of Ilexol Acetate. To a solution of 21.0 g of Fraction IV in 100 ml of benzene, was added 25 ml of acetic anhydride, and the resulting solution was

refluxed for 2 hrs. on a water-bath, after which the reaction mixture was concentrated *in vacuo* to remove benzene. The resulting concentrate was poured onto ice-cooled water to yield a mixture of crude acetate of m.p. 210–218°, which was filtered, washed with water, and then dried.

On fractionation with ethanol of this mixture, there remained a fraction of m.p. 284–288° (1 g) almost insoluble in ethanol, which after recrystallizations thrice from ethyl acetate, crystallized as rhombic plates (0.9 g: 0.07%), and melted at 290–291°, un-depressed on admixture with an authentic specimen of ilexol acetate[2]. The infrared spectra of this acetate and ilexol acetate were found to be superimposable one another, indicative of their identity: $[\alpha]_D^{14}$ −32.28°(c, 0.557). The reported value[1] of rotation $[\alpha]_D^{23}$ +22.29°($CHCl_3$, c, 0.045) was found to be errone-ous.

Anal. Found: C, 81.75; H, 11.21. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

Ilexol. A quantity of 0.3 g of ilexol acetate was refluxed for 2 hrs. with ethanolic KOH-solution (3%) to yield free alcohol, which after recrystallizations thrice from ethanol afforded needles (0.28 g), melting at 204–206°, undepressed on admixture with an authentic specimen of ilexol[2]. $[\alpha]_D^{14}$ −29.05°(c, 0.860, pyridine). The reported value of rotation[1] $[\alpha]_D^{23}$ +16.81°($CHCl_3$, c, 0.075)).

Ilexol Benzoate. To a solution of 0.18 g of ilexol in 1 ml of pyridine was added 1 ml of benzoyl chloride to yield a purplish-red solution with slight evolution of heat. After being left at room temperature over-night, the resulting reaction mixture was poured onto an amount of ice-cooled dil. H_2SO_4-solution to yield crude benzoate, which was thoroughly extracted with ether. The ethereal layer was washed successively with water, Na_2CO_3-solution, and water, and then dried. After removal of ether, there remained a yellowish mass, which was recrystallized several times from a mixture of ethyl-acetate and ethanol (1 : 1) to yield pure benzoate (0.17 g) in hexagonal plates, melting at 248–249°, either alone or on admixture with an authentic specimen of ilexol benzoate[2].

Anal. Found: C, 83.55; H, 10.20. Calcd. for $C_{37}H_{54}O_2$: C, 83.72; H, 10.25.

Hydrolysis with ethanolic KOH-solution (3%) of this benzoate afforded ilexol of m.p. 204–206°.

Isolation of α-Amyrin Benzoate. a) Fraction IV freed of ilexol acetate was deacetylated to yield a mixture of benzoates of m.p. 188–192°, which was

subjected to fractional crystallization from ca. 60 times its weight of a mixture of ethyl-acetate and ethanol (1 : 1).

From the fraction less-soluble in this solvent, there was obtained crude benzoate, which after several recrystallizations from ethanol, crystallized as long rectangular plates (13.5 g), and melted at 193–194°. b) To a solution of 47.5 g of Fraction III in 200 ml of benzene was added 50 ml of acetic anhydride, and the mixture was refluxed for 3 hrs.. After removal of benzene *in vacuo*, the solidified residue was dissolved in 100 ml of glacial acetic acid, and left in a refrigerator for several days. There separated scales, which were filtered, washed with water, dried, and recrystallized 6 times from ethanol to yield a mixture of acetates of m.p. 192–202°. The yield was 36.5 g.

Deacetylation-benzoylation of this mixture afforded a mixture of benzoates of m.p. 178–195°, which was subjected to fractional crystallization from a mixture of ethyl-acetate and ethanol (1 : 1). From the less-soluble fraction, there was obtained crude benzoate (12.0 g), which after several recrystallizations from the same solvent mixture, crystallized as long rectangular plates (3.2 g), and melted at 192–194°.

Melting points of both of these specimens of benzoates were undepressed on admixture with an authentic specimen of α-amyrin benzoate[2].

Anal. Found: C, 83.46; H, 10.18. Calcd. for $C_{37}H_{54}O_2$: C, 83.72; H, 10.25.

α-Amyrin. Hydrolysis with ethanolic KOH-solution (3%) of 0.5 g of α-amyrin benzoate of m.p. 193–194°, afforded free alcohol (0.45 g), which crystallized as needles from ethanol, and melted at 186–187°, undepressed on admixture with an authentic specimen of α-amyrin[2]. $[\alpha]_D^{14} + 83.75°$(c, 0.867).

Anal. Found: C, 85.28; H, 11.76. Calcd. for $C_{30}H_{50}O$: C, 84.44; H. 11.81.

α-Amyrin Acetate. Acetylation with pyridine-acetic anhydride of 0.3 g of α-amyrin of m.p. 186–187°, afforded crude acetate, which after several recrystallizations from ethanol crystallized as plates (0.3 g), and melted at 223–224°, undepressed on admixture with an authentic specimen of α-amyrin acetate[2]. $[\alpha]_D^{14} + 78.45°$(c, 0.794).

Anal. Found: C, 81.72; H, 11.20. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

Deacetylation of this acetate afforded α-amyrin of m.p. and mixed m.p. 186–187°.

Isolation of β-Amyrin Benzoate. a) From Fraction III freed of α-amyrin benzoate, there was ob-

tained crude benzoate, which after several recrystallizations from a mixture of ethyl-acetate and ethanol, crystallized and rectangular plates (0.3 g), and melted at 231–232°, undepressed on admixture with an authentic specimen[2]. b) From Fraction IV β-amyrin benzoate was isolated by treatment in just the same manner as above. The yield was 23.0 g. $[\alpha]_D^{14} + 96.67°$(c, 0.925).

Anal. Found: C, 83,41; H, 10.32. Calcd. for $C_{37}H_{54}O_2$: C, 83.72; H. 10.25.

Melting points of both these benzoates were undepressed on admixture one another.

β-Amyrin. Hydrolysis with ethanolic KOH-solution (3%) of 0.6 g β-amyrin benzoate of m.p. 231–232°, afforded β-amyrin (0.5 g) as needles from ethanol, melting at 196–197°, undepressed on admixture with an authentic specimen[3]. $[\alpha]_D^{14} + 85.64°$(c, 0.576).

Anal. Found: C, 84.16; H, 11.77. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

β-Amyrin Acetate. Acetylation with pyridine-acetic anhydride of 0.3 g of β-amyrin, afforded β-amyrin acetate (0.3 g) as prismatic needles from ethanol, and melted at 239–240°, undepressed on admixture with an authentic specimen[3]. $[\alpha]_D^{14} + 81.02°$(c, 0.757).

Anal. Found: C, 81.79; H, 11.09. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

Deacetylation of this acetate gave β-amyrin of m.p. and mixed m.p. 196–197°.

Isolation of Stearic Acid. An amount of 64.0 g of Fraction I was dissolved in 200 ml of ethanol, and left at room temperature for 2 days. There separated granular crystals, which after several recrystallizations from ethanol afforded scales, and melted at 70–71°, undepressed on admixture with an authentic specimen of stearic acid[4]. The yield was 12.5 g.

Anal. Found: C, 76.88; H, 12.35. Calcd. for $C_{19}H_{36}O_2$: C, 76.97; H, 12.24.

The acid amide of this acid (0.8 g) prepared in the usual manner, was recrystallized several times from ethanol to yield lamellar crystals (0.75 g), which melted at 108°, undepressed on admixture with an authentic specimen.

DISCUSSION OF THE RESULTS

a) The scientific names of original plants having hitherto been confirmed to be used for preparation of bird-lime, and b) the

3) K. Yagishita, This Bulletin, **20**, 97; 206 (1956).
4) K. Yagishita, S. Iseda, *J. Agr. Chem. Soc. Japan*, **29**, 964 (1955).

TABLE 1

THE OCCURRENCE OF TRITERPENOIDS AND β-SITOSTEROL IN VARIOUS SORTS OF BIRD-LIME

Original Plant	Family	Order	Unsaponifiable Constituents							Yield of Purified Resin (Bird-lime) Calculated on the Dried Plant Material
			α-Amyrin	β-Amyrin	Ilexol	Lupeol	Betulin	Taraxasterol	β-Sitosterol	
Loranthus europaeus Jacquin[5]	*Loranthaceae*	*Santales*								
Taxillus Kaempferi Danser[6,7]				+						fruits: 21.05%
T. Yadoriki Danser[6]										
Viscum album Linnaeus[8]										
var. *coloratum* Ohwi[6,9]				+		+			+	
B. alanophora elongata Blume[10]	*Balanophoraceae*	,,		+						
B. japonica Makino[3]			−	+	−	±	−	+	−	16.32%
Trochodendron aralioides Siebold et Zuccarini[11]	*Trochodendraceae*	*Ranales*	−	+	−	+	+	−	−	17.60%
Michelia compressa Sargent[12]	*Magnoliaceae*	,,								
Distylium racemosum Siebold et Zuccarini[13]	*Hamamelidaceae*	*Rosales*								
Ilex Aquifolium Linnaeus[14]	*Aquifoliaceae*	*Sapindales*	+							
I. Buergeri Miquel[15]										9.40%[16]
I. crenata Thunberg[17,22]			+	+	+	−	−	−	−	10.63%
I. integra Thunberg[2,22]			+	+	+	+	−	−	−	16.21%
I. Hanceana Maximowicz f. *rotundata* Makino[15,18]			+	+	−					15.44%
I. latifolia Thunberg[19]			+	+	−	−	−	−	−	19.12%
I. Oldhami Miquel[13,20]					−				+	1.38%
I. pedunculosa Miquel[12,13]										
I. rotunda Thunberg[12,13]										1.03%[21]
Osmanthus ilicifolius Standish[13]	*Oleaceae*	*Contortae*								
Cresentia Cujete Linnaeus[12]	*Bignoniaceae*	*Tubiflorae*								
Chondrilla juncea Linnaeus[23]	*Compositae*	*Campanulatae*						+(?)		
commercial " white bird-lime "[1,24]			+	+	+	+	−	−	−	
commercial " blue bird-lime "[2,25]			+	+	+,−	−	−	−	+	

5) J. Einleger, J. Fischer, J. Zellner, *Monatsh*, **44**, 277 (1923).

6) Y. Shimada, "On the Plants of the Family *Loranthaceae*, Naturally Occurring in Kyushu District", Botany (Kumamoto), No. 5, 1 (1955).

7) K. Yagishita, unpublished work.

8) a) K.H. Bauer, U. Gerloff, *Arch. Pharm.*, **274**, 473 (1936); b) J. Braunhauser, *Monatsh.*, **46**, 631 (1925).

9) Y. Obata, *J. Agr. Chem. Soc. Japan*, **17**, 222, 784 (1941).

10) M. Simon, *Monatsh.*, **32**, 89 (1911).

11) a) Y. Nishizawa, *J. Chem. Soc. Tokyo*, **41**, 1043 (1920); *J. Chem. Soc. Japan*, **43**, 154; 810 (1922); *ibid.*, **44**, 881 (1923). b) H. Yanagizawa, *J. Pharm. Soc. Japan*, **41**, 405 (1921); H. Yanagizawa, N. Nakashima, *ibid.*, **42**, 179 (1922); *ibid.*, **43**, 251 (1923). c) K. Sisido, S. Narita, *J. Ind. Chem. Soc. Japan*, **45**, 1187 (1942). d) K. Yagishita, This Bulletin, **21**, 77 (1957).

12) M. Watanabe, "*Sekai Jumoku Jii*" (A List of World Trees), rev. ed., Miura Shoten, Tokyo (1936).

13) R. Koketsu, *Bot. Mag. Tokyo*, **28**, 207; (in English) 161 (1914).

14) J. Personne, *Compt. rend.*, **98**, 1585 (1884); Ê. Jungfeisch, H. Leroux, *ibid.*, **147**, 862 (1908).

15) Y. Shimada, Private communication to the author.

16) K. Yagishita, unpublished work.

17) K. Yagishita, This paper.

18) K. Yagishita, unpublished work.

19) K. Yagishita, This Bulletin, **21**, 157 (1957).

20) K. Yagishita, unpublished work.

21) K. Yagishita, unpublished work.

22) N. Hirao, "*Abstracts of the Literature for the Chemical Constituents Occurring in Various Japanese Plants*", Vol, I, 391, Sendai Shoin, Sendai (1943).

23) a) A. Soler, J.C. Fernández, *Anales real. soc. espān. fis. y quim.* (Madrid), **50B**, 747 (1954); *Publ. inst. quim. "Alonso Barba"* (Madrid), **8**, 223 (1954); *Chem. Abstr.*, **49**, 11244 (1955). b) J.C. Fernández, *Anales univ. Murica* (Spain), **12**, 685 (1953–1954); *Chem. Abstr.*, **49**, 10992 (1955).

24) a) E. Divers, M. Kawakita, *J. Chem. Soc.*, **53**, 268 (1888); *J. Chem. Soc. Tokyo*, **9**, 153 (1889). b) Y. Nishizawa, *J. Chem. Soc. Japan*, **44**, 881 (1923). c) K. Sisido, S. Koga, I. Takahashi, *Saiensu*, **1**, 1 (1947).

25) K. Yagishsta, unpublished work.

occurrence in various sorts of bird-lime of triterpenoids and of β-sitosterol, are now, for ·convenience' sake, tabulated as follows.

1) Ilexol of $C_{30}H_{50}O$, m.p. 204–206°, which ·was first isolated from the unsaponifiable fraction of commercial "white bird-lime[1]", and later, from that of the bird-lime obtained from the bark of *Ilex integra* Thunberg[2], has now been confirmed to occur in that of the bird-·lime obtained from *Ilex crenata* Thunberg[17].

2) Commercial "blue bird-lime" is said to be originated from the bark of several species ·of plants, such as *Ilex latifolia* Thunberg, and .*I. crenata* Thunberg, etc. (Family: *Aquifoliaceae*) .as well as *Distylium racemosum* Siebold et Zuccarini (Family: *Hamamelidaceae*). In a-.greement with this fact, it has now been confirmed that ilexol is not always present in .a number of varieties of commercial "blue . bird-lime" available to date, thereby sug-.gestive of their diverse nature.

3) As reported previously[1], ilexol has been found to be levorotatory[17]: $[\alpha]_D^{14} - 29.05°$ ·(pyridine, c, 0.860). Contrary, however, to the ·description of the literature[1], it has now been confirmed that ilexol acetate is levorotatory[17]: $[\alpha]_D^{14} - 32.28°$ ($CHCl_3$, c, 0.577), indicative of the reported value of its dextrorotation[1]: $[\alpha]_D^{18} + 22.29°$ ($CHCl_3$, c, 0.045) is erroneous.

4) It is of interest to note that the presence ·of β-amyrin has been confirmed in all sorts (9) ·of bird-lime hitherto examined in detail in the literature.

5) The co-occurrence of α- and β-amyrin has now been confirmed in the unsaponifiable fraction of the bird-lime obtained from the bark of *Ilex crenata* Thunberg[17], just the same .as in the case of sorts of bird-lime obtained from the bark of *Ilex integra* Thunberg[2], *I. latifolia* Thunberg[19], *I. Hanceana* Maximowicz *f. rotundata* Makino[18] (Family: *Aquifoliaceae* as well as of commercial "white bird-lime[1]" and "blue bird-lime[2]".

6) Noteworthy is the fact that instead ·of α-amyrin (α-*Amyrin Group*), a) in the ¡bird-lime obtained from *Balanophora japonica*

Makino[3], taraxasterol (*Lupeol Group*) is present along with α-amyrin (α-*Amyrin Group*), and b) in the bird-lime obtained from *Trochodendron aralioides* Siebold et Zuccarini[11d], both betulin (*Lupeol Group*) and lupeol are present along with β-amyrin.

7) Of special interest is the fact that in the unsaponifiable fraction of the bird-lime obtained from *Ilex integra* Thunberg[2] ("white bird-lime")[1], ilexol of both unkown structure and configuration is co-existent with α-, β-amyrin, and lupeol.

8) In view of the present knowledge of taxonomy, "*Viscum album* Linnaeus *var. coloratum* Ohwi[26]" shoud be adopted as the valid scientific name of Japanese mistletoe (Japanese name: Yadorigi) instead of "*Viscum album* Linnaeus", a scientific name of European mistletoe.

Accordingly, the valid name of the original plant used by Y. Obata[9] in his previous work of "Constituents of Japanese Mistletoe" "should be" *Viscum album* Linnaeus *var. coloratum* Ohwi.

As already assumed by Y. Obata[9], it seems most likely that α- and β-viscol first isolated by K. H. Bauer and U. Gerloff[8a] from the bird-lime of European mistletoe, "*Viscum album* Linnaeus", are none other than β-amyrin and lupeol, respectively.

9) Also noteworthy is the fact that both Families of *Loranthaceae* and *Balanophoraceae* as well as both those of *Trochodendraceae* and *Magnoliaceae* belong to the Order of *Santales* and to that of *Ranales*, respectively, suggestive their close phylogenetic interrelationship a-mong themselves.

10) It has now been confirmed[15] that the bark of *Ilex Buergeri* Miquel and *I. Hanceana* Maximowicz *f. rotundata* Makino are being made use of preparing bird-lime at certain districts in Kumamoto Prefecture.

26) J. Ohwi, "*Flora of Japan*", Shibundo, Tokyo, 449 (1953)

Column Chromatographic Fractionation of Apricot Emulsin*

By Akira Baba

Shiga Agricultural Collega Shiga Pref. Japan

Crude emulsin of apricot (*Prunas armenica*) kernel was prepared by the method of tannin-fractionation. It was then purified by a fractional ammonium sulfate precipitation. The purified enzyme was further fractioned by adsorbing the enzyme on a CM-cellulose column and eluting it with the diluted McIlvaine's buffer solution. By this chromatography, six peaks of activities of β-glucosidase and β-xylosidase were developed. From one component of these peaks, petal-like crystals were obtained. The fractions thus obtained by chromatographic fractionation and crystallization were found differ with regard to the ratio of the β-glucosidase activity to the β-xylosidase one.

The specificity, purification and chemical nature of β-glucosidase, β-galactosidase and β-xylosidase have been investigated for many years by various Workers[1]. The complete purification of these enzymes, however, has not yet been reported.

Thus, Miwa *et al*[2] have shown that the β-glucosidase, β-galactosidase and β-xylosidase of apricot emulsin can at least, be partially separated by fractional ammonium sulfate precipitation and other fractional method, although these enzymes are often considered to be the different activities of a single enzyme. For fungal enzyme, Morita[3] has reported the separation of β-xylosidase from β-glucosidase, but has not succeeded in the isloation of β-xylosidase.

The homogeneity of the enzyme preparation thus reported, however, has not been shown by the above investigators, and the purification has usually been based on a relatively fractional method with several precipitating reagents.

On the other hand, Jermyn[4] employed the column chromatographic technique for examining the homogeneity of his stachybotrys atra β-glucosidase.

This paper deals with the chromatographic fractionation and crystallization of the enzyme in apricot emulsin.

EXPERIMENTAL

(1) **Preparation of Crude Emulsin** Ten kg of fresh apricot kernels was defatted with an oil expeller, and the cakes dried and finely powdered. By applying the purification method of Helferich *et al.*[5], 75 g of crude emulsin was obtained from this powder. In this procedure, zinc sulfate treatment and tannin-fractionation method were operated at room temperature (15–20°C) but the acetone treatment was performed in an ice box to prevent the inactivation of enzyme. In the last step of this process the enzyme precipitate was washed with petroleum ether and dried under reduced pressure. The purity of the enzyme preparation in this stage was observed by the electrophoretic analysis under the conditions indicated in the foot column of Fig. 1, and the existence of four or more components were recognized. On the other hand, specific β-glucosidic activity designated

* The present author uses the general term emulsin for expressing the enzyme system of apricot emulsin.

1) J.B. Sumner and K. Myrbäck, "The enzyme" Vol. 1, Part I, Academic Press 1950, p. 584-630 (cited by Veibel).

2) T. Miwa and K. Tanaka. "Symposia on enzyme chemistry" (Japan), 1949, p. 19

3) Y. Morita, *J. Biochem.*, **43**, 7 (1956).

4) M.A. Jermyn, *Australian J. Biol, Sci.*, **8**, 541 (1955).

5) B. Helferich, S. Winkler, R. Gootz, O. Peters, und E. Günther, *Z. physiol. chem.* **208**, 91 (1932). J.B. Sumner and K. Myrbäck, "The enzyme", Vol. 1, Part 1, Academic Press, 1950, p. 585 (cited by Veibel)

FIG. 1.   Electrophoresis of Crude Apricot Emulsin
(Hitachi electrophoretic apparatus)

Sample; after tannin-fractionation and dialysis: concn. of
protein; 2%: buffer; $\Gamma/2 = 0.05$, pH 4,8 acetate buffer: time;100
min.: tmp.: 5°C

as per mg protein was determined throughout the
purification process as shown in Table I.

(2) **Preparation of Purified Emulsin.** With the
procedure as shown in Table I, further purification
of crude emulsin was carried out. In this process,
collodion casing was employed for the dialysis of
enzyme solution, and it was found effective for the
removal of the yellow color impurity. At the end of
this purification process, the purity of this enzyme
preparation was investigated by the electrophoretic
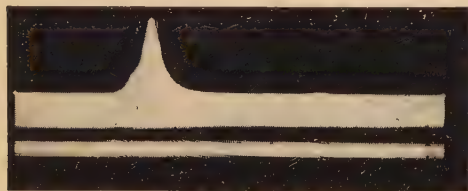analysis as shown in Fig. 2, and a slight separation

FIG. 2.   Electrophoresis of Purified Apricot Emulsin
(Spinco Model H electrophoretic apparatus)

Sample; after $(NH_4)_2SO_4$-fractionation, sp. act. 31 (β-glucosi-
dase): concn. of protein; 1.5%: buffer; $\Gamma/2 = 0.05$, pH 4,8 acetate
buffer; time; 74 min.: temp.; 5°C

of three or more components was observed although
the preparative separation of these components by
the electrophoretic apparatus was not accomplished.
The over-all purification designated as the specific β-
glucosidic activity was found to be approximately
300-fold in this stage.

Further purification of the enzyme with ammonium
sulfate was not successful. Therefore, the following
column chromatographic fractionation of the enzyme
was attempted.

(3) **Chromatographic Fractionation of Purified
Emulsin**

1. **Preparation of cation-exchanging CM-cellu-
lose** According to the briefly described procedure
of Sober *et al.*[6], cation-exchanging CM-cellulose was
prepared as follows: the mixture of 100 g cellulose

powder (Toyo Roshi Co., Ltd.) and one liter of 15%
sodium hydroxide solution was heated on a boiling
water bath for ten min.. After cooling, 20 g of
monochloroacetic acid was added to the mixture and
it was heated again for ten min.. The alkaline mix-
ture was neutralyzed with glacial acetic acid and
washed repeatedly with distilled water until the pH
of the washings became 5.6.

2. **Test for adsorption of β-glucosidase on CM-
cellulose by batch-operation** It could be shown
that the β-glucosidase in the dialyzed solution was
specifically adsorbed on CM-cellulose in the suitable
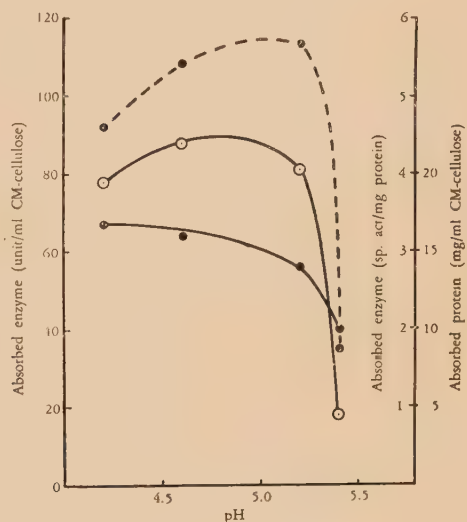pH (Fig. 3). In this test 5 ml of the dialyzed enzyme

FIG. 3.   Adsorption of β-Glucosidase on CM-Cellulose
(Batch operation)

Sample; ap. act. 4 (β-glucosidase): CM-cellulose; 1.5ml(sedi-
ment-volume): buffer; 10 times diluted McIlvaine's buffer
(0.02 M sodium phosphate-0.001M citric acid): room temp.
(30 ℃) —⊙— activity, —●— protein, - -●- - sp. act.
   β-glucosidase activity was determined by the method of
Sumner.[7] Protein was determined by the method of Gornall
et al[8]

solution (containing 185 units of β-glucosidase, sp. act.
4.0) was mixed with 2.5 ml of the diluted McIlvaine's
buffer solution (0.02 M sodium phosphate–0.001 M
citric acid) previously adjusted to various pH values

6)   H.A. Sober and E.A. Peterson, *J. Am. Chem. Soc.*, **76**, 1711
(1954).

7)   J. B. Sumner and G. F. Somers, "Laboratory Experiment in
Biological Chemistry" 1949, p. 144.

8)   A. G. Gornall, C. J. Bardawill and M. David. *J. Biol.*, *Chem.*
177, 751 (1949)

(4.0, 4.5, 5.0, 5.5). The buffered enzyme solutions were poured onto the CM-cellulose which had been sedimented in centrifuge tubes (sedimentary volume, 1.5 ml) and shaken immediately. After thirty minutes the adsorbents were centrifuged off. The quantities of protein and β-glucosidase activity units in these supernatant solutions were determined. From these values the amounts of adsorbed protein and enzyme were calculated. pH values of the supernatant solutions were also determined and the values were dotted in Fig. 3.

3. **Preparation of column** The above mentioned CM-cellulose was packed into the glass tube (diam. 1.45 cm, height 50 cm CM-cellulose column) with distilled water, and it was washed with distilled water until the washing lost its turbidity. Since the volume of the CM-cellulose was slightly decreased by washing, the volume was made up to the height of 50 cm. The packed CM-cellulose was buffered with 200 ml of diluted McIlvaine's buffer solution (pH 4.8, 0.02 M sodium phosphate–0.001 M citric acid).

4. **Adsorption of enzyme on CM-cellulose column** The purified enzyme was dialyzed against distilled water and buffered with the same volume of diluted McIlvaine's buffer solution (pH 4.8, 0.02 M sodium phosphate–0.001 M citric acid). This buffered enzyme solution (12.5 ml, containing 100 mg of protein, 3100 units of β-glucosidase) was poured onto the column.

5. **Fractional elution and assay of enzyme activity** As the eluent, diluted McIlvaine's buffer solutions adjusted to pH 4.8 and pH 8.0 (0.02 M sodium phosphate–0.001M citric acid) were prepared. The flask containing 200 ml of the pH 4.8 buffer solution was placed on a shelf, fitted in a height of 1.5 m from the upper end of the column, and connected to the column with a rubber tube. Then the buffer solution was continuously poured onto the column, and the pH of the buffer solution was slowly raised in steps from 4.8 to 7.0 by addition of the pH 8.0 buffer solution (when each 10 ml of the buffer solution flowed out from the column, the same volume of the pH 8.0 buffer solution was mixed into the flask). The effluent was collected in portions of 2 ml each and β-glucosidase and β-xylosidase activities were estimated by the following procedure. The quantity of protein in each fraction was determined by the method of Lowry et al[9]. For the measurement of β-glucosidase activity, the reaction mixture was prepared by mixing with 0.5 ml of 0.02 M salicin solution (salicin was dissolved with pH 4.4, 0.1 M

acetate buffer) and 0.04 ml of enzyme solution. After the incubation of the mixture at 30°C for one min., 0.5 ml of 0.1 N sodium hydroxide solution and 3 ml of dinitrosalicylic sugar reagent[10] were mixed successively, and the mixture was heated for five min. in boiling water. The optical density of this solution was taken in the Coleman universal spectrophotometer. By this procedure of enzyme assay, the unit of enzyme activity was not determined but the relative β-glucosidase activity was estimated. The β-glucosidase activity was measured by the same procedure except that O-cresyl β-D-xyloside (m.p. 161–2°) was employed as the substrate and the time of incubation was lengthened to thirty min..

By this column chromatography six (a, b, c, d, e and f) peaks of activities of β-glucosidase and β-xylosidase, respectively were observed as shown in Fig. 4, and the distinct shift of the ratios of β-glucosidase to β-xylosidase in each peak was recognized. The clear separation of β-glucosidase from β-xylosidase, however, did not occur.

(4) **Crystallization** As shown in Fig. 4 the effluent fractions were divided into six components (a, b, c, d, e and f), and each component was treated to crystallize with the procedure summarized in Table I.

In this way, petal-like crystals deposited from the component (a) and they were accompanied with a considerable quantity of amorphous matter. Most of the amorphous matter was separated from the crystals by centrifuging at low velocity of rotation. Fig. 5 shows the crystals from component (a). In other components no definite crystalline substance was found.

Recrystallization of the enzyme was not attempted, because of the insufficient amount of the crystals for this purpose.

(5) **Enzyme Activities of Crystalline Preparation** One drop of the crystalline suspension was dissolved with 10 ml of distilled water, and the β-glucosidase and β-xylosidase activities of one ml of this enzyme solution were measured by the procedure above mentioned. On the other hand, a small amount of the tannin-fractionated emulsin powder ("crude emulsin" in Table I) was dissolved with distilled water, and the insoluble matter was centrifuged out. This crude enzyme solution was diluted

9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, *J. Biol. Chem.* **193**, 265 (1951).

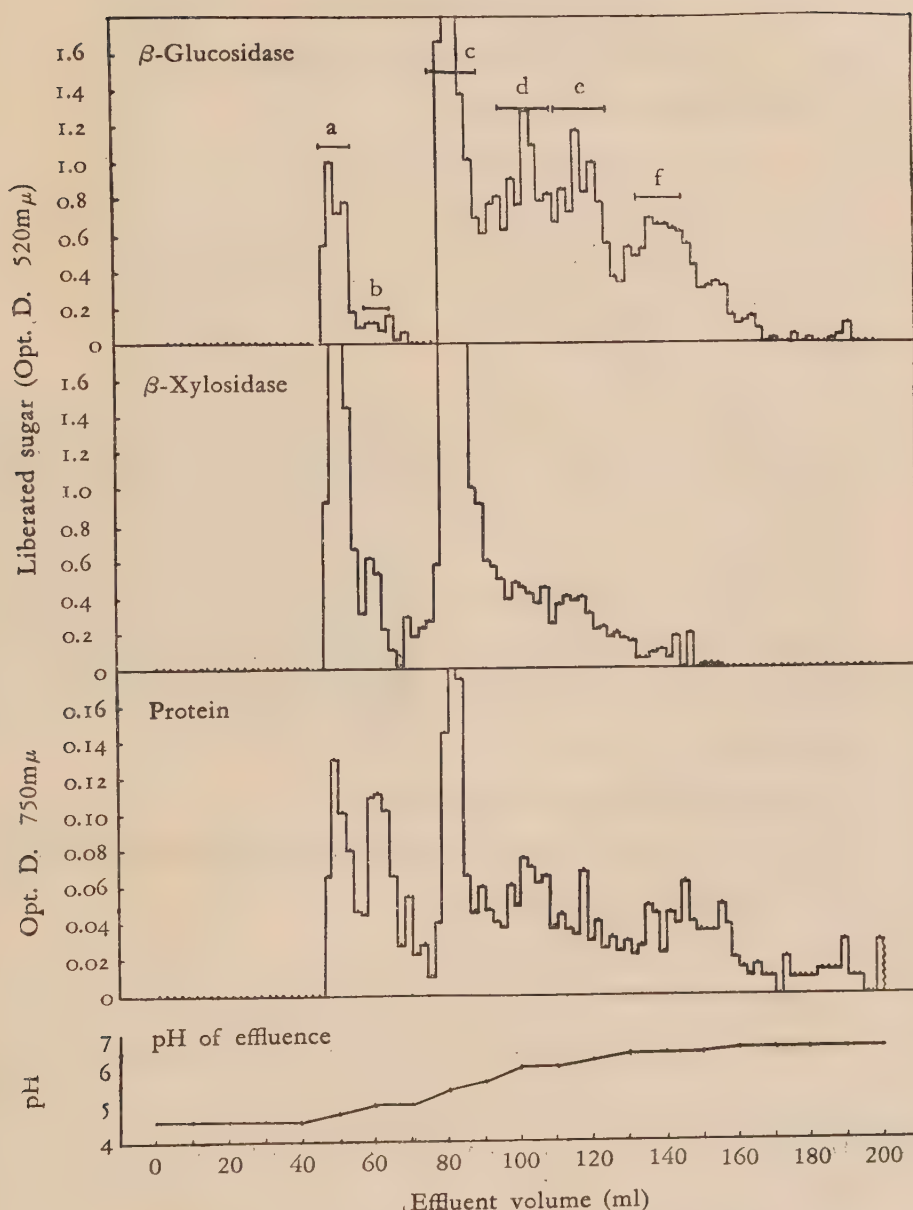(10) J.B. Sumner, *J. Biol. Chem.* **65**, 393 (1925).

FIG. 4. Chromatography of Purified Apricot Emulsin.

Sample; 100mg protein, sp. act. 31 (β-glucosidase): column; 50×1.45 cm (CM-cellulose): buffer for elution; pH 4.8-7.0 (pH 8.0, 10 times diluted McIlvaine buffer was added slowly to pH 4.8, 10 times diluted McIlvaine buffer): effluent rate; 3—10ml/hr.: temp.; 20—22° C: recovery of total protein; 80%.

with distelled water until one ml of this solution contains the corresponding amount of β-glucosidase activity to the above crystalline enzyme solution.

The β-xylosidase activity of this crude enzyme solution was measured, and compared with that of the crystalline enzyme solution. From this experiment a

TABLE I. PURIFICATION AND FRACTIONATION OF APRICOT EMULSIN

Powder of apricot seeds; sp. act. 0.1 (extd. with distd. H_2O)
↓

Tannin–fractionation; Helferich's method[5]
↓

Crude emulsin: Enzyme powder; 50g, ext. (1000ml distd. H_2O)
↓

Extract; filter with the aid of animal charcoal
↓

Filtrate; sp. act. 1, yield 100%, dialyze against distd. H_2O (collodion casing, 2 days)
↓

Dialyzate; filter with the aid of animal charcoal
↓

Filtrate; sp. act. 4, yield 91%
↓

1st $(NH_4)_2SO_4$-fractionation; 0.48-0.7 sat, pH 4.8, 30°C
↓

Precipitate; dissolve (minimum distd. H_2O), dialyze against distd. H_2O, (collodion casing, 2 days)
↓

Dialyzate; filter with the aid of animal charcoal
↓

Filtrate; sp. act. 6, yield 64%
↓

2nd $(NH_4)_2SO_4$-fractionation; 0.48-0.65 sat. pH 4.8, 30 C
↓

Precipitate; dissolve (minimum distd. H_2O), dialyze against distd. H_2O (collodion casing)
↓

Dialyzate; centrifuge, 4000 r. p. m., 20 min.
↓

Supernatant; sp. act. 23, yield 17%
↓

3rd $(NH_4)_2SO_4$-fractionation; 0.48-0.6 sat. pH 4.8, 30 C
↓

Precipitate; dissolved (minimum distd. H_2O), dialyze against distd. H_2O (collodion casing, 1 day)
↓

Dialyzate; centrifuge 4000 r. p. m. 20 min.
↓

Supernatant
↓

4th $(NH_4)_2SO_4$-fractionation; 0.5-0.6 sat. pH 4.8, 30 C
↓

Precipitate; dissolve (minimum distd. H_2O), dialyze against distd. H_2O, (collodion casing, 1 day)
↓

Dialyzate; sp. act. 31, yield 12%, add solid $(NH_4)_2SO_4$ (0.7 sat)
↓

Purified emulsin: Precipitate; (store in refrigerator), dialyze against distd. H_2O, nothing insol. matter
↓

Dialyzate
↓

Chromatographic fractionation
↓

Enzyme solution; (6 components), dialyze against 400ml of 0.7 sat. $(NH_4)_2SO_4$, outer soln change 3 times,
↓ centrifuge 7000 r. p. m. 15 min.

Precipitate; dissolve (minimum H_2O), dialyze against distd. H_2O (collodion casing, 1 day)
↓

Dialyzate; centrifuge 4000 r. p. m. 10 min.
↓

(Continuation of TABLE I)

↓

Supernatant ; add solid Na₂SO₄ (0.5 sat), add small amount of sat. (NH₄)₂SO₄

↓

Slight turbid solution ; add minimum amount of distd. H₂O until the white precipitate disapear in about
↓ 30 min.

Solution ; slowly condenced with silica gel (in refrigerator, 5 days)

↓

Crude crystal ; (appear from (a) component only), add 10 times vol. of 0.7 sat. (NH₄)₂SO₄, centrifuge 1000
↓ r.p.m. 2–3 min. (repeat 3 times), microscopic examination

Precipitate (purified crystal)

β-glucosidase activity was determined by the method of Sumner[7]
Protein was determined by the method of Gornall et al[8] or Lowry et al[9]
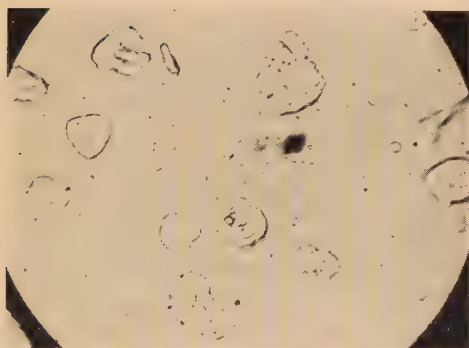Specific activity was designated as β-glucosidase units per mg protein.

FIG. 5.   Crystalls from Apricot Emulsin.
× 600

FIG. 6.   Comparison of the Activites of β-Glucosidase
and β-Xylosidase with Crude and Crystallized Pre-
paration.

crude enzyme; after tannin-fractionation: G; β-glucosidase
activity: X; β-xylosidase activity. Enzyme activities were
measured with the same procedure in the case of column chro-
matography

relatively strong β-xylosidase activity was proved in the crystalline preparation as shown in Fig. 6. However, the definite nature of the crystals still remains unsolved.

DISCUSSION

As mentioned above, crude apricot kernel emulsin was purified by the fractional ammonium sulfate precipitation with regard to the specific β-glucosidase activity as a criterion, but the complete purification of the enzyme by this procedure resulted in fail. The preparative separation of the components with the aid of Spinco Model H electrophoretic apparatus was not accomplished because of their extremely close mobilities. From the view point of these facts it is interpreted that apricot emulsin contains some components which have closely resembled physicochemical properties.

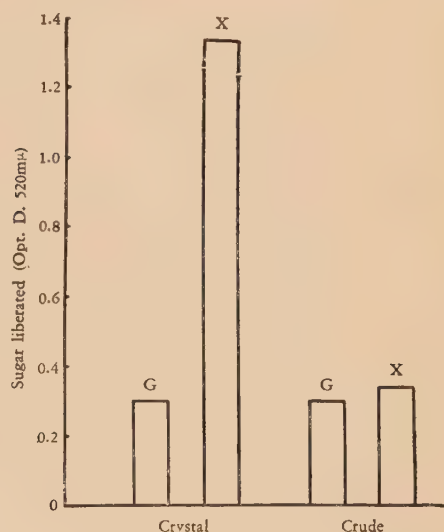When the purified enzyme preparation was chromatographed with CM-cellulose column, it was apparently separated in six components, and the crystalline substance was obtained from component (a) although the other components were not crystallized. Therefore, the chromatographic fractionation seemed to be effective for the separation of the crystalline substance.

As to the question whether β-glucosidase and β-xylosidase of apricot emulsin is com-

pletly separated, the author could not isolate any perfectly single enzyme in this study, but the distinct shift of the ratio of β-glucosidase to β-xylosidase was effected by the column chromatography. The separation of the two enzymes was not complete even after crystallization. However, it is noteworthy that the crystals indicated a relatively strong β-xylosidase activity.

On the other hand six peaks of activities of β-glucosidase and β-xylosidase were developed by the column chromatography, but the difference in these fractions has not yet been determined.

Studies on the Nutrition of Lactic Acid Bacteria

Part VI.   An Improved Method for the Microbiological Determination of Niacinamide

By Reijiro KODAMA

The Laboratory of the Institute for Fermentation, attached to Takeda Pharmaceutical Industries, Ltd. Osaka, Japan

An assay method for the quantitative determination of niacinamide found in natural materials based on its essential nature for *Lactobacillus fructosus*, a new species of lactic acid bacteria, is presented.   The values obtained here are reproducible within ±6%.   Recoveries of niacinamide added to the test materials could be made within ±6%.

INTRODUCTION

Up to the present, either *Shigella paradysenteriae*[1] or *Leuconostoc mesenteroides ATCC 9135*[2], has been used for the purpose of microbiological determination of niacinamide contained in natural materials.   *Shigella paradysenteriae* responds not only to niacinamide, but also to niacin, and a number of other related compounds, so that the value obtained can only be taken as "equivalents to niacinamide". On the other hand, samples have to be hydrolyzed prior to assay with sulfuric or hydrochloric acid in order to convert niacinamide to niacin, because *Leuconostoc mesenteroides* responds to niacin, but not to niainamide at low concentrations.

As previously reported, *Lactobacillus fructosus*[3], a new species of lactic acid bacteria, requires niacinamide as an essential growth-factor.   From this point of view, this organism may be suitable for that purpose.   In this paper, an assay method for the microbiological determination of niacinamide is presented.

EXPERIMENTAL

Organism.   The organism used, was *Lactobacillus fructosus Strain 353*.   Stab cultures were carried out in yeast extract-peptone-glucose-tomato juice agar (0.5% yeast extract, 0.5% peptone, 2% glucose, 40% tomato juice, 2% agar).   These stabs were prepared from previous stock cultures at bi-weekly intervals.   After transferation, the cultures were incubated at 36° for forty-eight hours, and then held at a low temperature.

Basal Medium.   The composition of the basal medium is shown in Table I.   While fructose, as reported in the previous paper[4], is essentially required for prompt and heavy growth of *Lactobacillus fructosus*, glucose stimulates the growth of this organism in the presence of fructose.   Consequently, both fructose and glucose are used as the carbon sources in the basal medium.   The possibility that unknown factors of a stimulatory nature may have an effect on the growth seems unlikely, in view of the facts that this organism grows luxuriantly on continued subculture in the basal medium to which niacinamide has been added. Indeed, *p*-aminobenzoic acid, acetate, fumarate, B_{12}, ribonucleic acid, desoxyribonucleic acid, ribonucleotide, ribonucleoside, thymine, cytosine, hypoxanthine, thymidine, pimelic acid, choline, inositol, glutathion, orotic acid and amino acids, are all ineffective.

1)   Harris Isbell, Jerald, G. Wooley, R.E. Butler, and W.H. Sebrell, *J. Biol. Chem.*, **139**, 499 (1941).

2)   B.C. Johnson, *J. Biol. Chem.*, **159**, 227 (1945); W.A. Krehl, J. De La Huerga, C.A. Elvehjem, and E.B. Hart, *J. Biol. Chem.*, **166**, 53 (1946).

3)   R. Kodama, *J. Agr. Chem. Soc. Japan*, **30**, 219 (1956).

4)   R. Kodama, *J. Agr. Chem., Soc. Japan*, **30**, 224 (1956).

Adenine sulfate	200 γ	Ascorbic acid	2 mg
Guanine hydrochloride	200 γ	Tween 80	10 mg
Xanthine	200 γ	L-Tryptophan	1 mg
Uracil	200 γ	Glucose	100 mg
Thiamine hydrochloride	5 γ	Fructose	200 mg
Riboflavin	10 γ	L-Asparagine	2 mg
Ca-pantothenate	5 γ	Bacto Vitamin Free Casamino Acids	50 mg
Biotin	0.05 γ	Salts A*	0.1 ml
Pyridoxal hydrochloride	5 γ	Salts B*	0.1 ml
Folic acid	1 γ	Adjusted to pH 6.4	

* Prepared according to Snell and Wright[5]

Niacinamide Standards. A solution containing 100 γ of niacinamide per ml, was freshly prepared on the days of assay.

PROCEDURE

Assays were carried out in 16 × 160-mm test tubes. Two and a half-ml of the basal medium which were prepared two-times the concentration of the final strength medium were pipetted into each tube; then, the standard niacinamide solution and solutions for analysis were added to the tubes. Triplicate tubes containing zero, 0.025, 0.05, 0.1, 0.2, 0.35, 0.5, 1.0 γ of niacinamide were set up for the standard curve. The samples for assay were set up at increasing levels of concentration estimated to contain the amount of niacinamide between 0.025 and 0.25 γ. The contents of all the tubes were then diluted to 5 ml with distilled water. The tubes were plugged with cotton and autoclaved under a steam pressure of ten pounds for ten minutes. After cooling to room temperature, they were ready for inoculation.

Inoculum. Inoculum for the assay tubes was prepared by transferation from the stock culture to a sterile tube of the basal medium to which 1.0 γ of niacinamide had been added. The inoculum was incubated at 36° for twenty-four hours before use. The cells from an inoculum cultured for twenty-four hour's were centrifuged out aseptically. After being washed twice with 0.85% saline, they were resuspended in saline. Then, the resulting suspension was diluted to 1 : 100. One drop of this suspension was added to each assay tube. The tubes were incubated at 36°, for approximately forty-eight hours.

5) Esmond E. Snell and Lemuel D. Wright, *J. Biol. Chem.*, **139**, 675 (1941).

Measurement of Response to Niacinamide. Growth was measured turbidimetrically by a Coleman spectrophotometer, of which the optical density is expressed as the difference between the readings of inoculated and not-inoculated tube.

RESULTS

Standard Curve. The response to the added niacinamide is shown in Fig. 1. A standard curve, similar to Fig. 1 must be obtained with each set of assays. The niacinamide content of each unknown tube was then read from the standard curve. From these values, the niacinamide content of the sample was calculated. The average of the values obtained at several levels on the curve within the assay limits was used. (Table II).
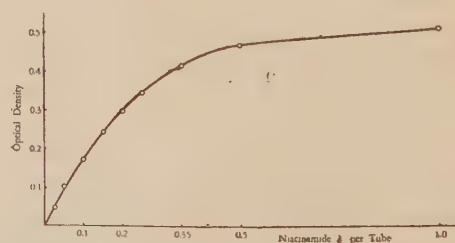

FIG. 1. Response of *Lactobacillus fructosus* to added niacinamide.

Preparation of Samples for Assay. Ten per cent aqueous solution of Armour's Soluble Beef and dried extract of yeast, were prepared for assay. A clear filtrate of canned-tomato juice was used as the sample. The samples

TABLE II
NIACINAMIDE CONTENT OF SOME NATURAL
MATERIALS AT VARYING ASSAY LEVELS

Material	Amount per Assay Tube	Niacinamide Found	Niacinamide Content
	ml	γ	γ per ml
Malt Extract	0.2	0.078	0.39
	0.1	0.037	0.37
			(0.38)
Liver Extract	0.005	0.160	32.0
	0.002	0.067	33.5
	0.001	0.034	34.0
			(33.17)
Yeast Extract	0.005	0.125	25.0
	0.002	0.049	24.5
	0.001	0.024	24.0
			(24.5)

all inactive. Nicotinuric acid, however, had 0.02% of the activity of niacinamide. On the other hand, niacinamide and Coenzyme-I has equal activity on their molar basis, so that the values obtained with this organism could not be taken as an absolute measure of niacinamide.

Recovery and Reproducibility. Assays on several materials to which niacinamide had been added, showed that the added niacinamide could be recovered within a region of ±6% (Table III).

Assays performed on the same materials at

TABLE III
RECOVERY OF ADDED NIACINAMIDE

Material	Amount per Assay Tube	Niacinamide Found per Tube	Niacinamide Added per Tube	Value Expected per Tube	Value Found per Tube	Recovery
Malt Extract	0.2 ml	0.081 γ	0.050 γ	0.131 γ	0.135 γ	103.1%
	0.1	0.041	0.050	0.091	0.095	104.4
Liber Extract	0.002	0.074	0.050	0.124	0.120	96.7
	0.001	0.035	0.050	0.085	0.080	94.1
Yeast Extract	0.002	0.055	0.050	0.105	0.102	97.1
	0.001	0.025	0.050	0.075	0.078	104.0

TABLE IV
REPRODUCIBILITY OF VALUES

Material	Values Obtained			Deviation of Individual Assay
	First Assay	Second Assay	Third Assay	
	γ per ml	γ per ml	γ per ml	%
Malt Extract	0.380	0.391	0.405	6.3
Liver Extract	33.17	34.33	35.1	5.5
Yeast Extract	24.5	25.33	26.16	6.4

of malt extract, liver extract, rice bran extract, spinach extract, and soy bean extract were prepared as follows: powdered malt, dried cow-liver (triangular), raw rice bran, fresh spinach, and the powder of raw soy bean were all extracted at 100° for one hour, with ten volumes of water and their filtrates were tested for analysis.

Specificity. Among niacin and the derivatives tested, only niacinamide was found to be active, specifically for the growth of *Lactobacillus fructosus*, while niacin, isonicotinic acid, picolinic acid, trigonelline, ethylnicotinate, quinolinic acid and coramine were

intervals varying from one to three weeks, showed that the values obtained on the given

TABLE V
NIACINAMIDE CONTENT OF VARIOUS
NATURAL MATERIALS

Material	Niacinamide Content
Malt Extract	0.391
Rice Bran Extract	0.077
Liver Extract	34.3
Tomato Juice	7.3
Yeast Extract	25.3
Spinach Extract	0.416
Beef Extract (Armour's Soluble Beef)	1.25
Soy Bean Extract	0.483

TABLE VI

COMPARATIVE VALUES OF NIACIN AND NIACINAMIDE OBTAINED WITH
L. ARABINOSUS, LEUC. MESENTEROIDES, and L. FRUCTOSUS

Material	L. arabinosus Total Niacin	Leuc. mesenteroides			L. fructosus Niacinamide
		Niacin		Niacinamide Total minus Free	
		Free	Total		
	γ per ml	γ per ml	γ per ml	γ per ml	γ per ml
Spinach Extract	0.7	0.27	0.58	0.31	0.42
Liver Extract	40.0	7.2	40.6	33.4	34.3
Yeast Extract	61.8	33.0	55.0	22.0	25.3
Tomato Juice	9.0	1.5	8.7	7.2	7.3

materials were reproducible to ±6% (Table IV).

Niacinamide Content of Various Natural Materials—The analytical values obtained with *Lactobacillus fructosus* are given in Table V.

Comparable results, obtained from selected samples, when assayed for niacinamide by the proposed procedure employing *Lactobacillus fructosus*, and according to the procedure of Krehl et al.[2] employing *Leuconostoc mesenteroides*, are given in Table VI. Both gave similar results, but the samples tested showed a somewhat higher value with *Lactobacillus fructosus* than with *Leuconostoc mesenteroides*.

DISCUSSION

It should not be concluded that niacinamide, when measured with *Lactobacillus fructosus*, is all composed of " free amide ", since this method does not distinguish the difference between the free amide and that bounds as cozymase. However, this method has several advantages, as shown in the following respects: (1) The test organism is non-pathogenic, (2) The response of *Lactobacillus fructosus* to niacinamide is only one-tenth of that of *Leuconostoc mesenteroides* to niacin, (3) The values obtained are independent of the effects owing to the acid hydrolysis of samples, and (4), The process is simple, since niacinamide is directly determined.

Studies on the Essential Oils of Tobacco Leaves

Part XIII. Basic Fraction (1)

By Isao Ōnishi and Kyoji Yamasaki

Tobacco Research Department, Central Research Institute, Japan Monopoly Corporation

The basic fraction contained in the essential oil of the Japanese Flue Cured Tobacco Leaf (before redrying) was investigated and 2,3′-dipyridyl was isolated and identified. While, on the other hand, the basic fraction of the essential oil of the aged Japanese Burley Tobacco Leaf was also investigated and nicotine was found to be the predominant constituent of that fraction. At the same time, a small amount of 2,3′-dipyridyl and myosmine was also isolated and identified.

Among the Acid-, Base-, Carbonyl-, Phenol-, and Neutral-fraction of the essential oil of aged Japanese Flue Cured Tobacco Leaf (JFCTL), the basic fraction has been considered to be interesting, having a characteristic fishy-odor resembling that of the Blending Department in a cigarette manufacturing factory. However, our investigation revealed that the basic fraction of the aged JFCTL contained very few basic nitrogen compounds, on the contrary, it contained a considerable amount of carbonyl compounds which came into the basic fraction because of their high solubility in water.

Nicotine, which is the predominant constituent of the bases contained in tobacco leaf, could not be found in the essential oil of aged JFCTL. This fact would prove that the basic compounds of aged JFCTL were not distillable because of its acidity (pH 5–5.3) and agreed with the results of Halle and Pribram[1] indicating that the essential oil of Hungarian tobacco leaf was N-free.

Subsequently, the basic fraction of the essential oil of JFCTL before redrying was investigated and a considerable amount of basic compound was isolated. The basic fraction of JFCTL before redrying had one spot (R_F value 0.92) on the paper chromatogram of Tso and Jeffrey's method[2] and the color reaction with PABA-BrCN[3] (BrCN only) gave a reddish-brown (yellow) color. After purification of the basic fraction for several times by the application of column chromatography, the basic compound was identified as 2,3′-dipyridyl by the ultraviolet absorption spectrum (Fig. 1.), mono-picrate (m.p. 151–2°) and di-picrate (m.p. 162–4°), which agreed with the results of Frankenburg[4]. However, the molecular extinction coefficient at 237 mμ in 0.2 N aqueous sodium hydroxide solution (ε 13.300) was slightly higher than those of Krumholz[5] (ε 11.400) and Frankenburg[4] (ε 11.600). The infrared spectrum of 2,3′-dipyridyl is shown in Fig. 2.

After the basic fraction of JFCTL essential oil was investigated, studies on the essential oil of the Japanese Burley Tobacco Leaf (JBTL) were conducted. As the pH values of the aged JBTL[6] and its steam distillate

1) W. Halle and E. Pribram, *Ber.*, **47**, 1394 (1914).

2) T.C. Tso and R.N. Jeffrey, *Arch. Biochem. Biophys.*, **43**, 269 (1951).

3) F. Kuffner and N. Faderl, *Mb. Chem.*, **87**, 71 (1956).
   In this paper, the color reaction with BrCN gave "ocker" but the author's result gave a yellow color

4) W.G. Frankenburg, A.M. Gottsho, E.W. Mayaud and T.C. Tso, *J. Am. Chem. Soc.*, **74**, 4309 (1952).

5) P. Krumholz, *J. Am. Chem. Soc.*, **73**, 3487 (1951).

6) J. Kobata et al, Sci. Papers Cent. Res. Inst. Japan Monop. Corp., **96**, 40 (1956).
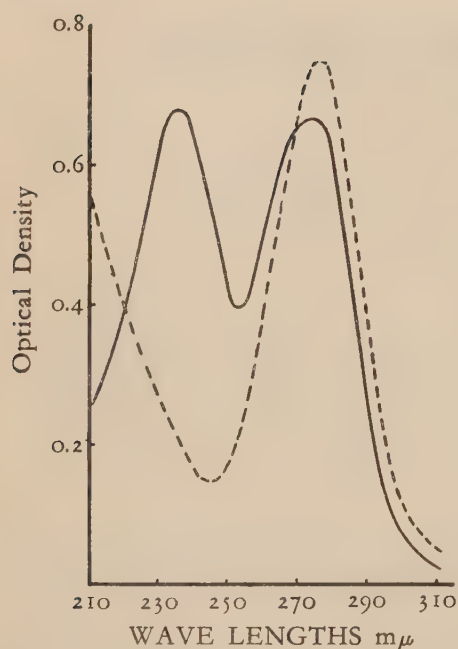
FIG. 1. Absorption Spectra of 2,3'-Dipyridyl, Obtained from Unaged Tobacco Leaf.
—— In 0.2 N aqueous NaOH (concentration, 8 mg of Dipyridyl in one liter), ... in 0.3 N aqueous HCl (concentration 6.9 mg of Dipyridyl in one liter).

were comparatively higher than those of JFCTL (see Table I.), a considerable amount of nicotine and other distillable minor alkaloids were steam distilled in the procedure of preparation of JBTL essential oil.

In order to remove nicotine, which was

the predominant constituent in the basic fraction of JBTL essential oil, the basic fraction was azeotropically distilled by the Smith's method[7] and the minor alkaloids remaining in the distillation residue were investigated.

It was presumed that the minor alkaloid fraction mainly consisted of two basic compounds, one of which was estimated to be 2,3'-dipyridyl by the infrared spectrum, ultraviolet spectrum and paper chromatography.

The aqueous solution (50 cc) of the minor alkaloid fraction was adjusted to pH 3.5 and extracted with ether, employing a liquid-liquid extractor to remove 2,3'-dipyridyl, by Frankenburg's method[4].
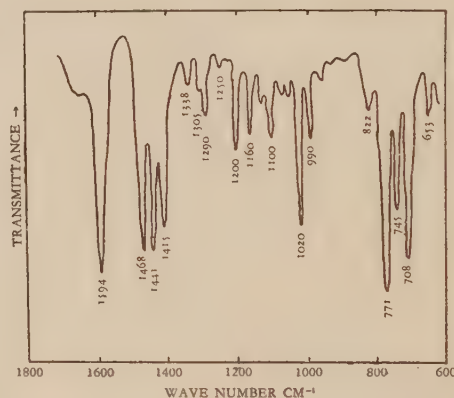
The extraction residue was adjusted to

FIG. 2. Infrared Spetrum of 2,3'-Dipyridyl, Obtained from Unaged Tobacco Leaf. (liquid).

TABLE I

PHYSICAL AND CHEMICAL CONSTANTS OF THE ESSENTIAL OILS OF THE JAPANESE
BURLEY AND FLUE CURED TOBACCO LEAVES

	JBTL	JFCTL	
	After Aging	Before Redrying	After Aging
Yield of Essential Oil (%)	0.398	0.133	0.215
pH value of Steam Distillate	6.8	5.5	5.5
Specific Gravity (d_{20}^{20})	1.001	0.984	0.829
Reflax Index (n_D^{20})	1.5170	1.4798	1.4131
Acid Value	0	76.81	—
Saponification Value	could not be determined	119.32	—
Ester Value	—	42.51	—

7) C.R. Smith, *Ind. Eng. Chem.*, **34**, 251 (1942).

pH 14, then extracted with ether again. One

alkaloid, which was isolated from this ether extract, was identified as myosmine by comparison of its infrared spectrum[8] and picrate (m.p. 185°) with those of authentic myosmine.

The yield of 2,3′-dipyridyl and myosmine were 0.11% and 0.03% for the basic fraction of JBTL, respectively.

2,3′-Dipyridyl

Myosmine
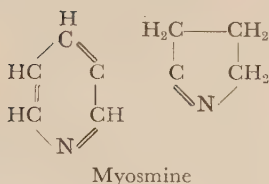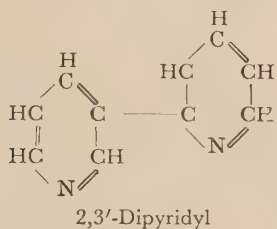
EXPERIMENTAL

2.A. Basic-Fraction in the Essential Oil of JFCTL Before Redrying.

2.A.1. Preparation of Basic-Fraction. The ethereal solution of the essential oil (179.36 g)[9] obtained from JFCTL before redrying was extracted three times with a 500-ml portion of 3% aqueous sulfuric acid solution saturated with sodium chloride. The sulfuric acid solution was alkalized, extracted with ether and the ethereal solution was dried over anhydrous potassium carbonate. After the removal of ether, 1.423 g of the basic fraction was obtained, which corresponded to 0.79% for total essential oil.

2.A.2. Isolation of Base. In order to remove the non-basic compounds contained in the basic fraction, 0.992 g of the basic fraction was eluted thoroughly with ether on a column of silicic acid-Celite mixture (2 : 1 by weight, 45 g, 17 cm × 2.5 cm dia.). The packing material, which absorbed the basic compounds, was suspended in 300 ml distilled water and this suspension was alkalized, then extracted with ether and the ethereal extract was dried over anhydrous potassium carbonate. Removal of ether left a crude basic fraction (232.7 mg) which contained a considerable amount of dark

colored resinous substances. In order to remove the dark colored impurities, the crude basic fraction was liquid-chromatographed by Houston's method[10]. About 60 g of starch was dry-packed in a column (2.5 cm dia.) and saturated with n-butanol, then eluted with n-hexane to remove the excess of n-butanol. On the top of this column, the crude basic fraction was placed and eluted with n-hexane. Almost all of the basic compounds were eluted with about 200 ml of n-hexane and the n-hexane solution was dried over anhydrous potassium carbonate. After the removal of n-hexane, 75.9 mg of the purified basic compound was obtained and it was then purified two more times by the same method.

2.A.3. Identification of 2,3′-Dipyridyl. Dipicrate of the basic compound was easily obtained from saturated aqueous solution of picric acid and recrystallized from hot water (m.p. 162-4°). *Anal.* Found: C, 43.55; H, 2.30; N, 18.39. Calcd. for $C_{22}H_{14}N_5O_{14}$; C, 42.99; H, 2.28; N, 18.24. On repeated recrystallization of di-picrate from hot water, mono-picrate (m.p. 151-2°) was obtained. *Anal.* Found: C, 49.59; H, 3.16; N, 18.12. Calcd. for $C_{16}H_{11}N_5O_7$; C, 49.87; H, 2.86; N, 18.18. These results (melting point, micro-analysis) and the ultraviolet absorption spectrum of the basic compound agreed well with those of 2,3′-dipyridyl, as reported by Frankenburg[4] and Krumholz[5].

2.B. Basic Fraction in the Essential Oil of JBTL.

2.B.1. Preparation of Basic Fraction. From 124 kg of cut Burley Tobacco Leaf (Smoking leaf, first grade, 1954 crop, grown in the Tohoku-district, Japan) 493.72 g of essential oil was obtained. The ethereal-solution of JBTL essential oil was extracted four times with 500-ml portion of 3% aqueous sulfuric acid solution, which was saturated with sodium chloride. The fourth extract (Fraction b.) presented a strikingly dark-brownish color in comparison with Fraction a., which consisted of the first to third extracts. Consequently, both fractions were investigated separately.

Both fractions were alkalized, then extracted with ether and the ethereal extracts were dried over pottasium carbonate. Removal of the ether left Fraction a. (243.719 g) and Fraction b. (7.387 g).

2.B.2. Identification of a Base in Fraction a. (Nicotine). It was presumed that this fraction consisted mainly of nicotine by paper chromatography. The di-picrate (m.p. 221-3°) from this fraction showed

8) C.R. Eddy and A. Eisner, *Anal. Chem.*, **26**, 1428 (1954).
9) I. Onishi and K. Yamasaki, This Bulletin, **21**, 86 (1957).

no depression upon admixture with the authentic nicotine di-picrate (m.p. 222–4°).

2.B.3. Separation of Bases in Fraction b. By paper chromatography it was found that this fraction contained a considerable amount of nicotine. In order to remove nicotine, Fraction b. was treated with azeotropic distillation by Smith's method[7], and 3.214 g of nicotine was yielded. After the distillation residue was alkalized, it was then extracted with ether and then, the ethereal extract was dried over anhydrous potassium carbonate. After removal of the ether, minor alkaloid-fraction (1.821 g) was left dark colored by impurities. Then, this fraction was purified by liquid chromatography employing a starch column as described previously for the purification of the base in JFCTL before redrying (2.A.2.).

It was estimated that this fraction consisted mainly of 2,3′-dipyridyl by its infrared spectrum and paper chromatogram. Consequently, the aqueous solution (ca. 50 ml) of this fraction was adjusted to pH 3.5, and then extracted with ether in a liquid-liquid extractor for about 24 hours. From the ethereal extract one basic compound (288 mg, Fraction b–1.) was obtained, which was already estimated to be 2,3′-dipyridyl. The extraction residue was alkalized, extracted with ether and this ethereal extract gave another basic compound (63.7 mg, Fraction b–2) after the removal of ether.

2.B.4. Identification of Fraction b–1. (2,3′-Dipyridyl). The ultraviolet absorption spectrum of Fraction b–1. agreed well with that of 2,3′-dipyridyl which was reported by Frankenburg[4] and Krumholz[5], and the mono-picrate (m.p. 151–2°) showed no depression upon admixture with the mono-picrate of 2,3′-dipyridyl (m.p. 151–2°) which was isolated and identified from the basic fraction of JFCTL essential oil.

2.B.5. Identification of Fraction b–2. (Myosmine). The infrared spectrum of Fraction b–2. agreed well with that of myosmine, reported by Eddy and Eisner[8], and the di-picrate (m.p. 185–5.5°) showed no depression upon admixture with the authentic di-picrate of myosmine (m.p. 185°)[10].

RESULTS AND DISCUSSION

2,3′-Dipyridyl was isolated and identified from the basic fraction of the essential oil of JFCTL before redrying. This compound was found to be present in many kinds

of tobacco[11,12], and Frankenburg[4] reported that 2,3′-dipyridyl, which was isolated from Pennsylvania Seedleaf tobacco, was the transformation product of nicotine during fermentation. As the pH value of JFCTL is between 5–5.3, it might be assumed that basic compounds were not steam-distilled in the procedure of preparation of essential oil; so really, nicotine was not to be found. However, the fact that 2,3′-dipyridyl was isolated and identified from the essential oil of JFCTL before redrying contradicts the general aspects.

On the other hand, 2,3′-dipyridyl could not be found in the aged JFCTL. It might be supposed that 2,3′-dipyridyl would have decreased during the aging stage.

In the Burley tobacco leaf, the alkaloids were steam distilled, and especially, a considerable amount of nicotine was distilled, as the pH value of the Burley tobacco leaf was between 6–6.5. In addition to myosmine and 2,3′-dipyridyl, which were both isolated and identified in the essential oil of JBTL, other minor alkaloids might be contained in the Burley tobacco leaf. However, less distillable alkaloids, such as nornicotine etc, would not come into the steam distillate in the general procedure of the preparation of essential oil.

10) This myosmine di-picrate was obtained through the courtesy of Dr. A. Eisner by the good services of Mr. E. Wada, member of the staff of our Tobacco Research Department.

11) H. Kuhn and H. Bühn, *Fachl. Mittl. Österr. Tabak-Regie*, 1956, Heft **1**, 6.
12) R.N. Jeffrey and T.C. Tso, *Agr. Food Chem.*, **3**, 681 (1955).

Studies on the Essential Oils of Tobacco Leaves

Part XIV.   Phenol Fraction (4)

By Isao Ōnishi and Katsumi Yamamoto

Tobacco Research Department, Central Research Institute, Japan Monopoly Corporation.

The caustic alkali soluble-fraction contained in the essential oil of Japanese Burley tobacco leaf (JBTL) was investigated.   Guaiacol, phenol, eugenol, *p*-allylpyrocatechol and *m*-cresol were isolated and identified.   The total amount of phenolic compounds was strikingly larger, amounting to more than twenty times as much as that of the Japanese Flue Cured tobacco leaf (JFCTL).   Guaiacol, the medium constituent in the essential oil of JFCTL, was found to be the predominant constituent exsisting in that of JBTL.   Besides these phenols, small amounts of benzaldehyde, 5-methylfurfural and 5-hydroxymethylfurfural (as carbonyls); as well as other   higher fatty acids were isolated from this fraction.

In our previous papers[1], the caustic alkali soluble-fraction (CASF), contained in the essential oil of JFCTL, both before redrying and after aging, were investigated and the presence of phenol, guaiacol, eugenol and *m*-cresol (as phenols); salicylaldehyde and *o*-hydroxyacetophenone (as phenolic carbonyls); *α*-pyrrylmethylketone, acetaldehyde, benzaldehyde, crotonaldehyde, *m*-toluldehyde and *p*-anisaldehyde (as carbonyls); lauric acid, myristic acid and palmitic acid (as higher fatty acids) has been reported.

Among these compounds, a striking decrease of phenols and a comparative increase of phenolic carbonyls during the aging stage were clarified in the previous papers[1].

The same procedures were undertaken on the essential oil of JBTL in this study.   The carbonyls were derived into 2,4-dinitrophenyl-hydrazones (2,4-DNPH), and separated by column chromatography.   5-Hydroxymethyl-furfural, benzaldehyde and 5-methylfurfural were isolated, and identified by their mixed melting points and infrared spectra.   After removal of the carbonyls from CASF, phenols were separated from higher fatty acids by means of a steam distilling procedure.   The phenols obtained were derived into 3,5-dinitrobenzoates and separated by column chromatography according to the White and Dryden method[2].   Consequently, guaiacol, phenol, eugenol, *p*-allylpyrocatechol and *m*-cresol were isolated, and these were identified by their mixed melting points and infrared spectra.

On the other hand, the phenols were derived into azo-compounds, coupled with diazotized *p*-nitroaniline, and the contents of each constituents were determined by a spectrophotometric method, succeeded by a chromatographic separation on an almina column.

It was a noteworthy fact that the amount of phenols found in the essential oil of JBTL was strikingly larger amounting more than twenty times to that of JFCTL.   Furthermore, guaiacol was comprised by half of the total phenols and the content of guaiacol amounted to about thirty times as much as that of JFCTL.

p-Allylpyrocatechol, though its content was

1) I. Ōnishi and K. Yamamoto, This Bulletin, **19**, 148 (1955); *ibid*, **20**, 70 (1956); *ibid*, **21**, 90 (1957).

2) J.W. White Jr. and E.C. Dryden, *Anal. Chem.*, **20**, 853 (1948).

TABLE I

COMPARISON OF THE CAUSTIC ALKALI SOLUBLE COMPONENTS IN THE ESSENTIAL
OILS OF JBTL AND JFCTL

| | JBTL | | JFCTL | | | |
| | After Aging | | Before Redrying | | After Aging | |
	mg/kg of leaf	% for total	mg/kg of leaf	% for total	mg/kg of leaf	% for total
Carbonyls	0.79	0.54	0.25	0.18	0.59	0.58
Phenols	29.76	20.32	15.18	11.02	1.05	1.03
Higher Fatty Acids	115.91	79.14	122.34	88.80	100.13	98.39
Total	146.46	100.00	137.77	100.00	101.77	100.00

TABLE II

COLUMN CHROMATOGRAPHIC SEPARATION OF PHENOL-3,5-DINITROBENZOATES
OF ESSENTIAL OIL OF JBTL

Band No.	Compound	m.p. (°C)	Color
6	Unknown	172–3	Cream Yellow
5	Guaiacol	140.2	Yellow
4	Phenol	144.0	Colorless
3	m-Cresol	186.7	Colorless
2	p-Allylpyrocatechol	150.2	Cream Yellow
1	Eugenol	130.0	Yellow

Band numbers indicate the order of elution.
Adsorbent: Silicic acid + Celite (2 : 1).
Column: 200 × 36 mm.
Developing solvent: 5% Ether in n-Hexane.
Borders of separations were detected by U.V. and chromatostrips.

not so high, was isolated and identified as a new phenol in the essential oil of tobacco leaves.

EXPERIMENTAL

From 124 kg of cut Burley tobacco leaf (Smoking leaf, first grade, 1954 crop, grown in the Tohoku district, Japan), a comparative amount of CASF was separated. As reported previously[1], carbonyls were also separated from the ethereal-solution of CASF by shaking with an aqueous solution of sodium bisulfite. After the removal of ether, the residual fraction was steam distilled so as to avoid any fatty acids being distilled out. From the steam distillate, phenols were obtained. Table I shows the yields of these fractions, in comparison with those of JFCTL.

2.A. Carbonyls in the Caustic Alkali Soluble-Fraction. By the method as reported in the previous paper[1], 2,4-DNPH of the carbonyls (326.2 mg) was liquid-chromatographed and benzaldehyde (m.p. 242°, 18 mg), 5-methylfurfural (m.p. 223°, 7 mg) and 5-hydroxymethylfurfural (m.p. 194°, 16 mg) were

separated in accordance with their eluting order. Identifications of these compounds were conducted by their mixed melting points and infrared spectra.

2.B. Phenols in the Caustic Alkali Soluble-Fraction. The phenols separated by steam distillation from higher fatty acids, were derived into azo-compounds, coupled with diazotized p-nitroaniline, and developed by paper chromatography. However, as the separation of spots on the chromatogram was not so satisfactory another alliquot of the phenols was derived into 3,5-dinitrobenzoates and separated by column chromatography.

2.B.1. Separation and Identification of Phenols. Following the method of White and Dryden[2], the column was packed with a mixture of silicic acid and Celite (2 : 1, by weight) which contains a small amount of Rhodamine 6 G (80 mg in 450 g of adsorbent) and developed by n-hexane which contains 5% of ether. Six bands were separated. Removal of the solvent from the ether extract of each band left crystal and it was recrystallized from n-hexane. The results are shown in Table II.

The cream-yellow needles, obtained from Band 2, melted at 150° and showed no depression upon admixture with the authentic p-allylpyrocatechol (m.p. 152–3°). Furthermore, the infrared spectrum of the needles completely agreed with that of the authentic p-allylpyrocatechol. Besides, the other phenols, which were preliminarily isolated and identified in the essential oil of JFCTL, were identified by their mixed melting points and infrared spectra with the authentic compounds.

2.B.2. Determination of Contents of Phenols. The phenols (2.B.) were derived into azo-compounds, then separated by column chromatography. The "Brockmans" alumina was employed as the adsorbent and monochlorbenzene as the eluting solvent. Six bands were separated and the amounts of these bands, after cutting off the band and extraction with an aqueous solution of 75% isopropyl alcohol containing 1% of aqueous solution of N-sodium hydroxide, were determined by the Beckman Spectrophotometer. In Table III the results obtained are shown, compared with those of JFCTL.

2.C. Separation and Identification of Higher Fatty Acids in the Caustic Alkali Soluble Fraction. The steam distilling residue of the above 2.B. was derived into 2,4-dinitrophenylhydrazide and identified by a reverse phase chromatography, as described in the previous paper[1]. Consequently, palmitic acid, myristic acid and lauric acid were identified.

RESULTS AND DISCUSSION

The CASF of the essential oil of JBTL was investigated. Eugenol, guaiacol, phenol, m-cresol and p-allylpyrocatechol (as phenols); benzaldehyde, 5-methylfurfural and 5-hydroxymethylfurfural (as carbonyls); lauric acid, myristic acid and palmitic acid (as higher fatty acids) were isolated and identified.

The essential oil of JBTL contains about twenty times the amount of phenols in comparison with that of JFCTL. This fact might intensely affect the smoking aroma and taste of JBTL. On the other hand, the amounts of higher fatty acids and carbonyls contained in the CASF of JBTL, are similar but slightly larger than that of JFCTL.

The carbonyls, separated from the CASF of the essential oil of JBTL, mainly comprized of some resinous substances, and isolation of the

carbonyls was rather difficult. Consequently, other carbonyls found in the said fraction of JFCTL, such as salicylaldehyde, o-hydroxyacetophenone and acetaldehyde etc., could not be isolated.

It is noticed that no predominant constituent was found in the phenols of aged JFCTL (see Table III.). However, in the case of JBTL, guaiacol was proved to be the pre-

TABLE III

CONTENTS OF PHENOLS IN THE ESSENTIAL OILS OF JBTL AND JFCTL (Both are Aged)

	JBTL		JFCTL	
	mg/kg of leaf	% for total	mg/kg of leaf	% for total
Guaiacol	11.73	49.22	0.38	36.19
Phenol	6.37	26.74	0.41	39.05
Eugenol	3.27	13.72	0.26	24.76
p-Allylpyrocatechol	2.15	9.02	—	—
m-Cresol	0.24	1.00	—	—
Unknown	0.07	0.29	—	—
Total	23.83	100.00	1.05	100.00

dominant constituent, comprized half of the total phenols and its content amounted to about thirty times as to that of JFCTL.

On the other hand, a striking decrease of phenols was clarified in the case of JFCTL during the aging stage, which amounted to one-fourteenth[1]. Provided that the changes of phenols were similar in both JFCTL and JBTL, although no details are yet obtained at present, the JBTL before redrying might contain much more phenols than that of after aging. Anyway, the high content of phenols seems to strengthen the smoking aroma and taste of tobacco leaf.

p-Allylpyrocatechol is one of the di-valency phenols newly isolated from tobacco essential oils. This compound had been reported in Java betel leaf oil[3], but we have no report at hand concerning its presence in other plants. Although the chemical structure of p-allylpyrocatechol is similar to that of eugenol, the odor resembles that of cresol. In general, di-valency phenols are considered

3) E. Guenther, "The Essential Oils", Vol. II, D. Van Nostrand Co., Inc., N.Y. (1949), p. 513.

to be unstable in the natural plant tissue, and they easily suffer from oxidation and polymerization resulting brownish resinous substances. Consequently, the presence of free di-valency phenols in the essential oil of JBTL, together with the fact that JBTL contains a larger amount of phenols than JFCTL, might suggest some tendency which indicates that JBTL does not easily suffer from oxidation. This presumption was supported by the fact that the essential oil of JBTL contains a very small amount of acids in comparison to that of JFCTL[4].

The Chromatographic Purification of Yeast Invertase by an Ion-Exchange Resin Method and Some Properties of the Enzyme Obtained

By Takehiko Yamamoto, Junichi Kumada
and Teruo Sawai

Institute of polytechnics, Osaka City University; Biological Institute, Aichi Gakugei University

The purification of yeast invertase was attempted by application of the chromatographic method using Duolite C–10, a sulfonic acid cation exchange resin. This method was found to be extremely simple in process and significantly effective for the improvement of purity of the enzyme, compared with those other methods reported, hitherto. In the present paper, the procedure of the purification and some properties of the enzyme obtained thereby, are described, and some discussion of the implications is presented.

A great number of papers have been published on the purification of yeast invertase. The methods employed hitherto are mostly those that repeat the procedure of precipitating the enzyme with solvents such as alcohol[1] and acetone[2] or with chemicals such as tannic acid[3], uranium acetate[4], picric acid[2], etc. and adsorbing the enzyme on kaolin[3], aluminium hydroxide[5,6], bentonite[2], tricalcium phosphate, etc..

In the purification of yeast invertase, the procedure to deliver the enzyme free from a polysaccharide, known as yeast mannan, appears to be the most difficult. In fact, isolation of the enzyme in a completely free state from the polysaccharide has not yet been attained, and thus, the important problem as to whether the yeast invertase is a polysaccharide-conjugated protein or not, remains unsolved[5,7,8].

Formery, one of the authors in studying on yeast amylase found, that a certain kind of polysaccharide which persistently adheres to the enzyme preparate can be easily removed by the application of ion-exchange resin method, and this led the authors to the suggestion that an yeast invertase preparate freed from polysaccharide might be obtained by the application of ion-exchange resin method. Complete removal of the polysaccharide from active yeast invertase, however, has not yet been achieved in the experiment, but the method was found to be useful for purification of the enzyme. In the present paper, the procedure of purification by the ion-exchange resin method and some properties of yeast invertase obtained thereby, are described.

MATERIALS AND METHODS

1. Preparation of Yeast Invertase Solution Applied to the Ion-exchange Resin Method. Ten kg of pressed brewer's yeast was suspended in 12 l of 1.5% sucrose solution, containing 0.2% ammonium sulfate and 0.2% monobasic potassium phosphate and centrifuged after standing overnight at 10° to 15°C (pH 5.2–5.4). The precipitated yeast was then mixed with 500 ml of toluene and autolyzed at 25°C, the pH

1) C. O'Sullivan and F.W. Tompson, *J. Chem. Soc.*, **57**, 834 (1890).
2) M.Adams and C.S. Hudson, *J. Am. Chem. Soc.*, **65**, 1359 (1943).
3) R. Willstätter, K. Schneider und E. Wenzel, *Z. physiol. Chem.*, **151**, 1 (1926).
4) M. Jacoby, *ibid*, **30**, 135 (1900).
5) Ed. H. Fischer et L. Kohtès, *Helv. Chim. Acta*, **34**, 1123 (1951).
6) L. Michaelis und M. Ehrenreich, *Biochem. Z*, **57**, 70 (1910).
7) J.B. Sumner and D.J. O'Kane, *Enzymologia* **12**, 251 (1948).
8) A.I. Oparin and M.S. Bardinkaya, *Izvest. Akad. Nauk. S S S.R., Ser. Biol.*, **1955**, No. 2, p. 3

adjusting up to 7.0 with sodium hydroxide. After two days, the autolysate was filtered through a filter paper and the filtrate was acidified to pH 4.2 with glacial acetic acid. A thick precipitate formed thereby was removed by centrifugation and the supernatant liquid, after dialysis against distilled water for 3 days at 6°C using a cellophane bag (5.5 l), was adopted as the starting enzyme solution for the purification experiment. The invertase activity of the solution was 198 units on the assay method described below.

2. Assay Methods of Activity and Purity. Enzyme reaction for activity assay, in which 1 ml of invertase solution was added to 2 ml of 3.75% sucrose solution containing M/10 acetate buffer of pH 4.4 was carried out at 20°C for three min.. The amount of sugar inverted was determined as glucose, by a modification of the Fehling-Lehmann-Schoorl method[10] (After three min. of reaction, 1 ml of the solution of Rochelle salt and sodium hydroxide was added to the reaction mixture in order to deactivate the enzyme, followed by 1 ml of the copper sulfate solution. The mixture, being kept in boiling water for ten min. and subsequently cooled, was titrated with N/20 sodium thiosulfate, after the addition of 1-ml of each of the potassium iodide and the sulfuric acid solution). In the present paper, the enzyme activity that forms 1 mg of reducing sugar as glucose under the condition was defined as one unit. Reducing sugar formation, ranging in an amount from about 0.5 to 6 mg, was found to run perfectly parallel to the enzyme concentration, on this assay test.

The polysaccharide present in enzyme preparate was determined by electrophotometry using anthrone as the color developer[11], and the amount expressed as glucose.

Nitrogen was assayed by the micro-Kjeldahl method, but sometimes, the distillated ammonia obtained, was subjected to electrophotometrical analysis at 440 mμ, after letting a color develop with Nessler's reagent (The distillate collected into dilute sulfuric acid was brought up to a certain volume, making the final concentration of sulfuric acid to M/100, then to 10 ml of the solution was added 0.5 ml of Nessler's reagent.). The amount of nitrogen, suitable for measurement by this method was about 5 to 30 γ per 10 ml.

For the investigation of the electrophoretical properties of the enzyme, the Tiselius electrophoresis apparatus (adopting the cylindrical lens, schlieren optical system) and the Svensson and Brattsten paper electrophoresis[12] apparatus with the continuous flow system were used.

3. Ion-Exchange Resins. Duolite A-2, an anion-exchange resin, for decolorization and the removal of some of impurities and Duolite C-10, a cation-exchange resin, for the chromatographic purification of the enzyme were used, respectively, after being pulverized to a particle size ranging from approximately 100 to 200 mesh.

RESULTS

1. Purification by the Ion-exchange Resin Method. The A-2 resin, which had been soaked in 3 N ammonia solution for several hours, was washed with distilled water, subsequently with N/10 hydrochloric acid. After once adjusted with M/20 phosphate buffer solution of pH 6.0, then washed with distilled water, the resin was added to the crude invertase solution in the ratio of one of the former to the five of the latter, by weight. The suspension, after being stirred for 15 min. at room temperature, was filtered through a Buchner funnel with the aid of a suction pump. By this treatment, the invertase solution became completely colorless, although no remarkable improvement in the purity of the enzyme was observed.

The A-2 resin-treated enzyme solution was then buffered at pH 3.2 with citric acid and dibasic sodium phosphate, in concentrations similar to those of the conditioning solution for Duolite C-10 resin. Meanwhile, the suspension of the C-10 resin, which had been soaked in 2 N sodium hydroxide solution for several hours, then washed with distilled water and subsequently with 0.5 N hydrochloric acid, was introduced into a glass tube. After settling the resin, the resin column was conditioned by flowing down the McIlvain's citric acid-dibasic sodium phosphate buffer of pH 3.2. Following this conditioning step,

9) T. Sawai, unpublished
10) A.W. van der Haar, "Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren", Verlag von Gebruder Borntrager, Berlin, 1920, S. 120.
11) T.A. Scott and E.H. Melvin, *Anal. Chem.*, **25**, 1656 (1953).
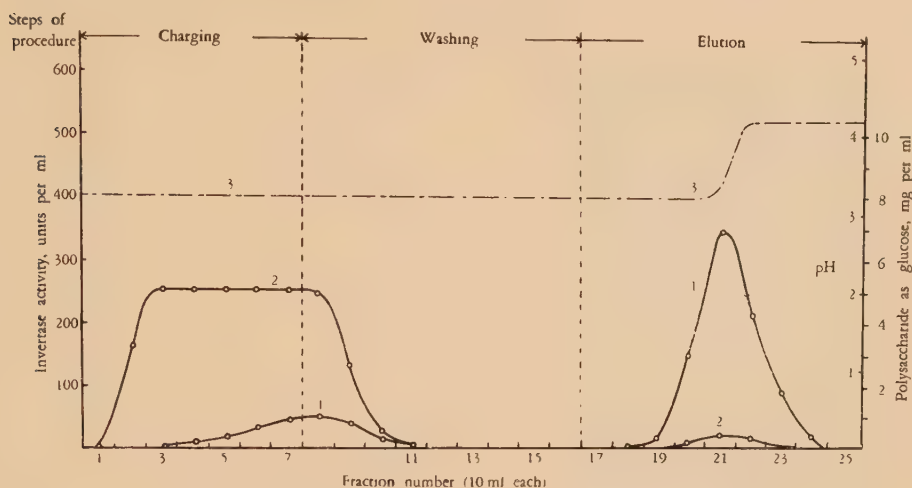12) E.L. Durrum, *J. Am. Chem. Soc.* **73**, 4875 (1951).

FIG. 1. Chromatographic Purification of Yeast Invertase Using a Sulphonic Acid Cation Exchange Resin "Duolite C–10".

Resin column, 1.75 cm dia.×9 cm depth; enzyme solution charged, see Table 1; curve 1, activity; curve 2, polysaccharide as glucose; curve 3, pH.

TABLE I

SOME DATA REFFERING TO FIG. 1.

	Enzyme, charged	Enzyme, eluted (Fraction No. in Fig. 1)		
		20	21	22
Total vol., ml	70	10	10	10
Total activity, units	10,430	1,510	3,100	2,350
Activity, units per mg N	339	6,020	5,550	4,850
mg polysaccharide as glucose	24.8	711	630	442

the buffered invertase solution was charged into the resin column and made to pass down the colum at a rate of 0.03 ml per ml resin per min.. Once the enzyme solution had passed through the resin, the column was washed with a buffer, whose constituents were the same as those used for the conditioning, and then developed with the McIlvain's citric acid-dibasic sodium phosphate buffer of pH 4.2, with the same velocity as at the charging step.

From the results shown in Fig. 1 and Table I, it will be seen that over ninety per cent of the polysaccharide in the original enzyme solution passes through into the break-through solution and washings while a major part of

the activity comes out into the eluate, of which the pH has been changed with the progress of elution, the purity of the enzyme being greatly improved on the basis of activity per mg of both nitrogen and polysaccharide. However, it was found that repetition of this ion-exchange resin method has little effect on further improvement of the purity of the enzyme.

The enzyme-holding capacity of the resin was shown to be approximately 500 units in activity per ml of the resin, with the column over 3 cm in height. But, in the experiment, it was often found that a small amount of the enzyme passed through the column appearing in the break-through solution, even at an early stage of the charging step, irrespective, to a certain extent, of the amount of enzyme solution charged thereto.

Change in flow velocity of the enzyme solution through the resin column at the charging step did not appear to have any remarkable influence upon the enzyme-adsorption efficiency, within the rate of 0.02 to 0.1 ml enzyme solution per ml resin per min.. This was found to be the case with

the elution step, further, in order to obtain a high purity-enzyme solution, finely fractioning of the eluate was more favorable than controlling the velocity of elution.

With regard to the type of buffer, few experiment were undertaken, the reason being that some kinds of buffer system intefere with the assay of enzyme purity. Acetic-acetate buffer was found unsuitable for this resin method. Change in concentration of the buffer used in the present paper, also had almost no significant effect on either adsoption or development efficiency, within the range of M/10 Na₂HPO –M/20 citric acid to M/2.5 Na₂HPO –M/5 citric acid.

On the other hand, temperature was found to have a marked effect on the activity of the enzyme, bringing a deactivation of the enzyme increasingly with the increase of temperature over 10°C. The effect is however,
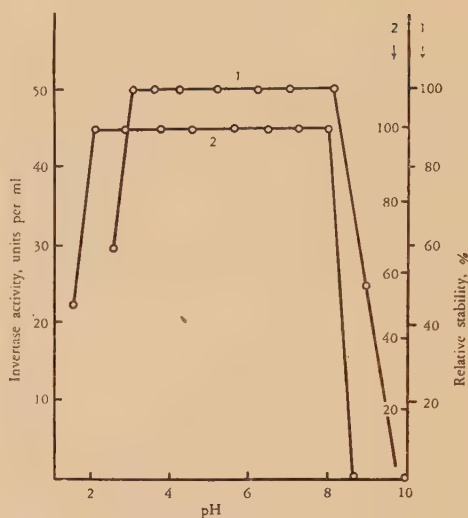
FIG. 2.   pH-Stability of Yeast Invertase (20°C, 24 hrs.).

Curve 1, purified enzyme; curve 2, unpurified one.

Incubation period, min. Invertase	60	30	15		60	30	15		Standard sugar, each 200γ
		purified				unpurified			
Total activity of the enzyme added, units		73				60			
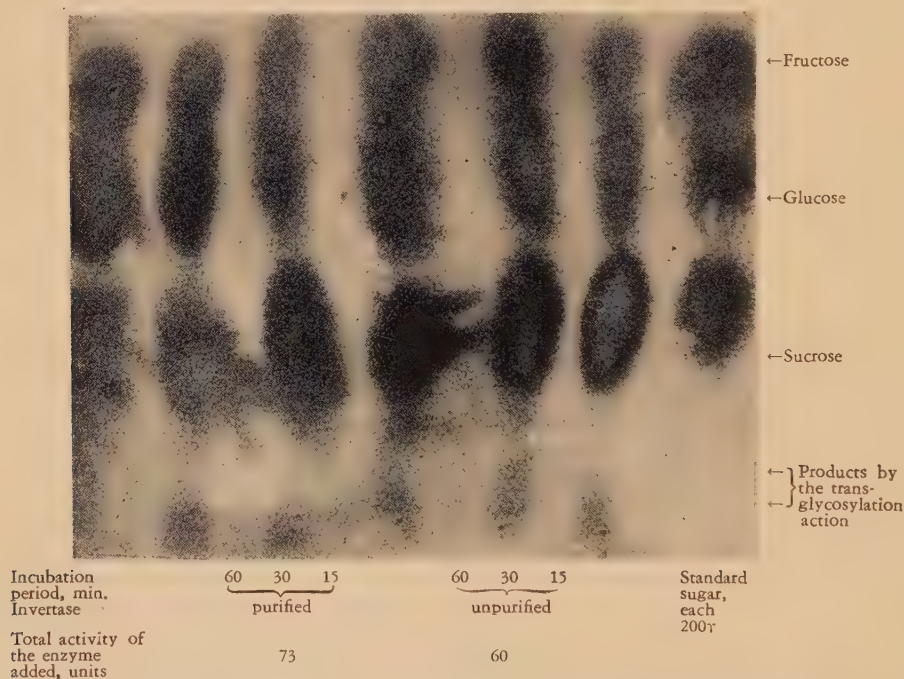
FIG. 3.   Action of Yeast Invertase on Sucrose :   Chromatograms of 10 μl of a Reaction Mixture
0.5 ml of enzyme solution and 5ml of 20% sucrose solution containing M/10 acetate buffer of pH 4.4 at 20 C, stopped the reaction by addition of phenylmercuric acetate (the final conc., M/500), multiple development by ascending method, developed the color by benzidine method.

purely temporary whilst the enzyme is held on the resin.

Besides the C–10 resin, Duolite CS–101 and Amberlite XE–64, both of which are carboxylic acid cation exchange resins, were also investigated for chromatographic purification of yeast invertase, but these two resin gave unsatisfactory results.

2. Some Properties of the Enzyme Obtained. The enzyme thus purified by the resin method was subjected to tests of stability, uniformity of enzyme action, ultraviolet absorption and electrophoresis. For the first three tests, enzyme solution of high purity obtained by the resin method was used, after it was dialyzed against distilled water for forty-eight hours in the cold using a cellophane bag.
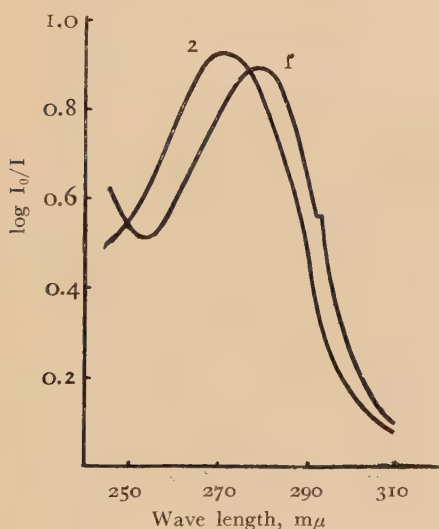
FIG. 4. Ultraviolet Absorption of Yeast Invertase.

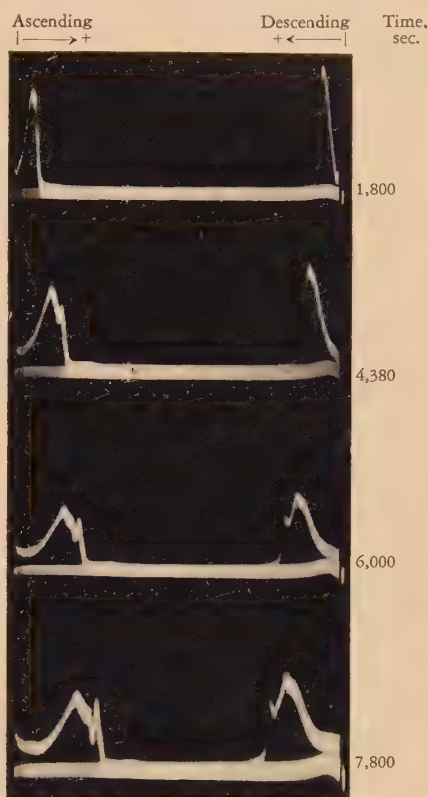| Enzyme	Curve 1 purified by the resin method	Curve 2 ppted. with 60% alc. from the Duolite A–2 resin-treated enzyme sol. and dissolved
Activity, units per ml	514	50
N, mg per ml	0.105	0.075
Polysaccharide as glucose, mg per ml	1.16	1.86
pH	5.0	5.0

FIG. 5. Electrophoretic Pattern of Yeast Invertase Purified by the Resin Method.

2750 u. activity, 0.63 mg N and 6.45 mg polysaccharide as glucose per ml; M/10 citric acid-M/5 Na2HPO4 buffer of pH 4.4, 18 mA, 118 V at 25°C.

It will be noticed from Fig. 2 that the pH-stability range of the enzyme migrates slightly towards the alkaline side as a result of purification by this method.

Concerning the uniformity of enzyme action, only the activities of peptidase, maltase and transglycosylase[13] were investigated. The activities of both peptidase and maltase were found to be entirely negative, whereas, the activity of transglycosylase was found to be almost in the same degree as that in the starting enzyme solution, at an equal level of invertase activity (Fig. 3).

The ultraviolet absorption spectra of the purified enzyme is shown in Fig. 4, indicating

13) J. Edelman and J.S.D. Bacon, *Biochem. J.*, **49**, 529 (1951).
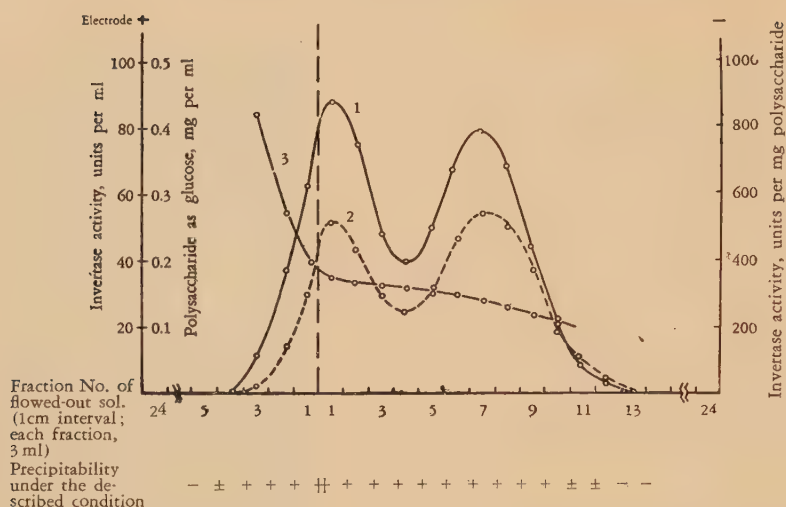
FIG. 6.   Distribution of Yeast Invertase in the Paper Electrophoresis by the Svensson and Brattsten's Flow System Method.

Enzyme charged, 7 ml (301 u. activity, 0.079 mg N and 0.808 mg polysaccharide as glucose per ml, this enzyme had been subjected to the Tiselius electrophoresis experiment (Fig. 5); M/50 citric acid-M/25 Na₂HPO₄ buffer of pH 3.2, 17 mA, 385 V/50 cm, 32°C, 48 hrs., the vertical broken line shows the place of the enzyme supplier (equipped at 40 cm high); curve 1, activity; curve 2, polysaccharide; curve 3, activity per mg polysaccharide.

that the absorption maximum lies at 278 mμ wave-length.

For electrophoresis experiments, the enzyme obtained by the resin method (total activity, 15,350 u/61 ml; purity, 512 u/mg polysaccharide and 5,700 u/mg N) was further treated as follows: It was first precipitated by the addition of acetone (final conc., 58%; pH 3.6; 6°C) and, after dissolving this precipitate in a M/10 citric acid-M/5 dibasic sodium phosphate buffer of pH 4.4, was dialyzed against the same buffer for twenty-four hours using a cellophane bag at 20°C (total activity, 9,625 u/3.5 ml; purity, 394 u/mg polysaccharide and 4,360 u/mg N).

As seen in the photographs, the purified enzyme was still found to contain at least two components, electrophoretically: one, being small in amount rapidly migrating towards the anode, and the other, being a major component moving very slowly. The enzyme solution tested with the Tiselius apparatus was then subjected to the Svensson and Brattsten paper electrophoresis and the result showed that two peaks exist on the distribution curve of enzyme activity that was obtained from the flowed-out solutions. By subjecting to the paper electrophoresis, the enzyme solution was found to become precipitable when treated by the addition of trichloroacetic acid (2 M), dibasic sodium phosphate (M/5) and citric acid (M/10) and heating at 100°C for five min.. With the flowed-out solution of the paper electrophoresis experiment, the amount of precipitate produced by that treatment appeared to run approximately parallel to the activity distribution curve.

DISCUSSION

From the experiment on the purification of yeast invertase by the ion-exchange resin method which gave a result, that where by a single procedure the purity of the enzyme was significantly improved as shown in Fig. 1 and Table I, being brought up to a level of 6,000 units and 700 units per mg of nitrogen and polysaccharide as glucose, respectively,

on the assay method described in the present paper, the resin method will be fully understood to be an excellent for purification of the enzyme. However, there appears to be a limit to the purity attainable by this method, since repetition of the method did not result in further purification. It may be necessary to make further investigations concerning the type of resin most suitable for the method. This also seems to be of importance to approach the problems concerning the nature of yeast invertase.

It has been reported that yeast invertase becomes unstable when it is purified[14], but in the authors' experiments the enzyme purified by the ion-exchange resin method was found to give heat-stability almost similar to that of the untreated one, with the exception of significant heat-sensitivity found in the enzyme held on the resin. The enzyme also seemed to be considerably stable in an electric field, because in the Tiselius electrophoresis experiment there was almost no loss of enzyme activity. The purified enzyme, however showed itself to be more sensitive to acetone or alcohol than the unpurified one.

With regard to the ultraviolet absorption of yeast invertase, the absorption maximum has been reported to lie in region, 265 to 275 mμ[8] or 265 mμ[14], leaving suspicion that there might be some contamination caused by purine or pyrimidine bases. With the enzyme purified by the present method, however, the absorption maximum was found to lie at 278 mμ, as in the case of many proteins.

The Tiselius electrophoresis experiment, on the other hand, gave the result that yeast invertase purified by the present method separates electrophoretically into two or more fractions, as reported by Fischer and his coworkers[14]. A similar result was also observed in paper electrophoresis by the Svensson and Brattsten method, showing that two peaks

14) Ed. H. Fischer, L. Kohtès et J. Fellig, *Helv. Chim. Acta.*, **34**, 1132 (1951).

exist on the distribution curve of enzyme activity. However, such a trend was not observed with the curve which traced enzyme activity per unit amount of carbohydrate for each fraction obtained there. This result seems to indicate that after all, yeast invertase might be freed from yeast mannan, although a strong affinity may exist between the enzyme and the polysaccharide.

SUMMARY

Purification of yeast invertase was attempted by means of chromatography using Duolite C-10, a sulfonic acid cation exchange resin, and some properties of the enzyme obtained thereby were investigated. The results obtained are as follows:

1. By this method a yeast invertase preparate can be purified to an extremely advanced state, especially in its polysaccharide content.

2. As a result of purification by the method, the pH-stability range of the enzyme migrates slightly towards the alkaline side. But, there occurs almost no change in the heat-stability of the enzyme.

3. Almost no change in transglycosylation activity of the enzyme is exhibited.

4. In ultraviolet absorption spectra, the absorption maximum of the enzyme shifts from about 270 mμ to 278 mμ, greatly increasing enzyme activity per unit of the extinction coefficient.

5. However, the purified enzyme was found to contain still two or more components, electrophoretically.

Electrophoretic Study of Protein Metabolism in Sweet Potato Infected with *Ceratostomella fimbriata**

By Takashi Akazawa, Yasuichi Umemura and Ikuzo Uritani

Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University, Anzyo, Aichi-ken, Japan

It was shown in previous papers that in sweet potato infected with *Ceratostomella fimbriata*, there occurred metabolic activations such as respiratory increase, polyphenols production, protein synthesis and organic phosphate accumulation. In this report on an electrophoretic experiment, forming a part of those studies, it is stated that the pattern of the protein components in the sound part, adjacent to the infected part of sweet potato attacked by the pathogen differs from that of sound sweet potato.

INTRODUCTION

We have described in previous papers[1,2] the effect of *Ceratostomella (C.) fimbriata* (black rot) infection on the respiration and metabolism of nitrogen and phosphorus of sweet potato. In the sound part of the tissues adjacent to the injured area, there occurred metabolic activations such as respiratory increase, protein synthesis and organic phosphate accumulation in response to the penetration of the pathogen. From this we have suggested that this active metabolism may indicate the stimulation of protoplasmic activity *in situ*.

It is conceivable, that not only a net-increase in the protein content, but also a change in the molecular structure of the protein in the sound tissues of infected sweet potato can occur. In order to attempt an elucidation of the mechanism in the alteration of protein metabolism in the infected sweet potato, the electrophoretic analysis of various protein fractions has been undertaken. Such experiments may be of value to reveal the resistance mechanism of infected plants, in general.

* Part 23 of Phytopathological Chemistry of Black Rot Sweet Potato.
1) Akazawa, T., and Uritani, I., *Nature*, **176**, 1071 (1955).
2) Akazawa, T., *Science*, **123**, 1075 (1956).

EXPERIMENTAL

(1) Sampling.—Sweet potato (variety: Okinawa No. 100) was divided into three groups; fresh, control (sliced and uninoculated), and infected tissues. A spore suspension of *C. fimbriata* was inoculated on slices, about 1.0 cm thick, which were then incubated at 25°C for two days. The surfaces of these slices were injured to a depth of about 0.5 cm. The control slices (cut, but not treated) were incubated under the same conditions, simultaneously. The sound part adjacent to the infected tissues, the corresponding part of the control tissues, and the central part of the fresh root were taken and subjected to the analysis described below.

(2) Extraction of Protein by Means of Phosphate Buffer. (Procedure A)—To 25 g of sweet potato tissues 30 ml of ice cold phosphate buffer (M/25 pH 7.2) was added, and it was homogenized for 15 min. in a cold mortar. The pH was adjusted to 7.2 with 1 N NaOH during the process, and the homogenate was thereafter centrifuged for 10 min., at 500 × g. The sediment was discarded out and the resulting supernatant was frozen overnight, then thawed and dialyzed overnight against phosphate buffer (M/25 pH 7.2), and the final supernatant was used in the electrophoretic analysis.

(3) Ammonium Sulfate Precipitation of Phosphate Buffer Extract. (Procedure B)—To 15 g of sweet potato tissues was added 15 ml of phosphate buffer (ionic strength (μ) 0.1, pH 7.2, containing 0.5% ascorbic acid), and it was homogenized and cen-

trifuged in the same manner as that mentioned in Procedure A. After a further 5 ml of phosphate buffer was added to the sediment, homogenization and centrifugation procedures were repeated. The volume of the supernatant was made up to 25 ml, and to a 20-ml aliquot of this fraction, saturated ammonium sulfate solution was added so as to attain a 75% saturation. After one hour, it was centrifuged at 7,000×g for 15 min., and the sediment was dissolved in 7 ml of the above-mentioned buffer (containing ascorbic acid). After adjusting the pH to 7.2, the solution was dialyzed overnight against the same buffer (not containing ascorbic acid), centrifuged for 15 min. at 8,500×g and the resulting supernatant used in the electrophoretic study. Each step of the procedure was carried out around 0°C.

(4) Extraction of Protein as Acetone Powder. (Procedure C)—To 15 g of sweet potato tissues was added 200 ml of purified acetone (precooled to −25° ~ −30°C), and homogenized vigorously for about 3 min. in a Waring blendor. During the procedure, the temperature wa sheld at −25°~−30°C. The resulting acetone powder was washed with 200 ml of cold acetone and 100 ml of cold ethyl ether successively, then finally dried over P_2O_5 in a vaccum desiccator. It was then eluted repeatedly with the phosphate buffer (μ0.1, pH 7.2 and containing 0.5% ascorbic acid), and made up to 15~16 ml. The solution was dialyzed against the phosphate buffer (not containing ascorbic acid), and the dialyzed solution was treated in the same manner as that described in the preceding section, so as to obtain the electrophoretic protein fraction.

(5) Electrophoretic Analysis.—The Shimadzu scanning electrophoretic apparatus was used. 200 v, 10 mA for 45~60 min., at 4°C.

(6) Determination of the Nitrogen Content of Protein Fraction.—After 4 ml of 10% trichloroacetic acid (TCA) was added to a 1 ml aliquot of the electrophoretic solution, it was centrifuged at 1,000×g for 10 min. Five ml of 5% TCA solution was added to the sediment; it was centrifuged and the final precipitate analyzed by the microkjeldahl method to estimate the amount of nitrogen.

(7) Measurement of Polyphenol Oxidase Activity.—Activity of polyphenol oxidase was determined manometrically in the conventional Warburg apparatus. Main cell: 2 ml of 0.01 M methyl caffeate and 1 ml of phosphate buffer (M/20, pH 6.0), side arm: 0.1 ml of enzyme solution (electrophoretic sample),

equilibration for 10 min., temperature 30°C.

(8) Determination of Polyphenols in the Ammonium Sulfate Precipitated Protein Fractions.—The ammonium sulfate precipitated protein fractions, were homogenized with cold acetone repeatedly. The resulting acetone solutions were analyzed to determine their amounts of polyphenols by the method shown in the preceding report[3].

RESULTS

1. We have failed to obtain a clear electrophoretic picture from the protein extract of Procedure A. This may be attributed to the following reasons.

(a) The extraction of protein was incomplete and the extracted electrophoretic fraction was diluted. Consequently, the electrophoretic pattern was obscure.

(b) As the polyphenol oxidase activity was higher, particularly in the case of the extract from the sound part of infected sweet potato, and also a large amount of polyphenols was attached to the protein molecules, the electrophoretic protein solution had a brownish appearance, even though it was prepared at a controlled low temperature. The descending pattern of electrophoresis could not normally be taken owing to incomplete transmission of light.

(c) A particlar fraction including " mitochondria " was precipitated into the sedimentable fraction during the high speed centrifugation.

2. Some difficulties have been observed, even in the case of Procedure B. By the addition of ascorbic acid to the preparative medium, the protein extract from the infected sweet potato remained colorless for a time. As a result, rapid handling of the ammonium sulfate preparation gave an almost white precipitate in each fraction. However, sudden browning occurred during the following thawing and dialysis procedures against phosphate buffer in case of the protein fraction from the infected sweet potato; furthermore, during the high speed centrifugation after

3. Uritani, I., *J. Agr. Chem. Soc. Japan*, **27**, 57 (1953).

TABLE I. THE AMOUNTS OF PROTEIN NITROGEN AND POLYPHENOLS

Procedure	Assayed substances	Sample	Healthy	Control	Infected
B	Protein nitrogen mg N	First extd. soln.	16.43	16.65	12.38
		Residue in centrifuge after dialysis	1.05	1.16	5.55
		Supernatant in centrifuge after dialysis (electrophoretic sample)	10.94	9.15	4.67
	Porlyphenols mg N	Ammonium sulfate pptd. fraction	5.0	6.0	10.0
C	Protein nitrogen mg N	Residue in centrifuge after dialysis	0.74	0.97	1.43
		Supernatant in centrifuge after dialysis (electrophoretic sample)	12.30	15.15	14.10

Fifteen g of each tissue was used for analysis.

dialysis a rather large amount of nitrogen compounds was sedimented in the fraction from the infected tissues (Table I). These results indicate that the electrophoretic patterns of the protein in infected sweet potato prepared by Procedure B were less clear than those of both fresh and control sweet potato. Some possible reasons for this difference are suggested. Firstly, in the course of ammonium sulfate fractionation, polyphenols are adsorbed on the protein molecules, being also co-precipitated with them. Since polyphenol oxidase itself is contained in the protein, it may oxidize the attached polyphenols rapidly in the presence of air to form quinones; these may combine with the protein molecules and allow denaturation to follow. Secondly, there is a large amount of ammonium ion which may react with the quinones to form Schiff's base, accelerating denaturation of the protein. The fact that the ammonium sulfate precipitated protein fraction of infected sweet potato contains a larger amount of polyphenols than the other two fractions, may fortify the above possibility (Table I). Furthermore, it is possible that the protein of infected sweet potato is more labile than the others, and subjected to the action of denaturing agents, such as the polyphenols and the quinones to a further degree.

3. Extraction as acetone powder (Procedure C).—By considering experimental difficulties encountered in the above two procedures, it was hoped that a more successful method could be applied to prepare the protein fraction of the infected sweet potato. The requirements were: a) to raise the nitrogen content of the electrophoretic sample, and b) in order to avoid denaturation of the protein, the removal of harmful effects of polyphenols or quinones. Preparation of the acetone powder and the following procedure have achieved this purpose, and the denaturation of the protein molecule has been prevented by treating the tissue $-25° \sim -30°C$. As shown in Table I, comparable nitrogen contents of the electrophoretic samples were obtained from each fraction. From the results of electrophoretic analyses of the protein fractions prepared by this procedure, which were carried out three times, it was clarified that the electrophoretic pattern of injured sweet potato was distinct from the other two fractions. Representative data are diagrammatized in Fig. 1. Electrophoretic patterns of the fresh sweet potato and control tissues are more or less similar and have one main peak, while contrarily, the infected sweet potato protein indicates two main peaks [A and B] (their patterns and mobility values are shown in Fig. 1). Although we must be cautious to conclude that this preparative method does not denature the protein melecule entirely, the activity of polypheonl oxidase, shown in Fig. 2, indicates that this rather labile enzyme was extracted in something

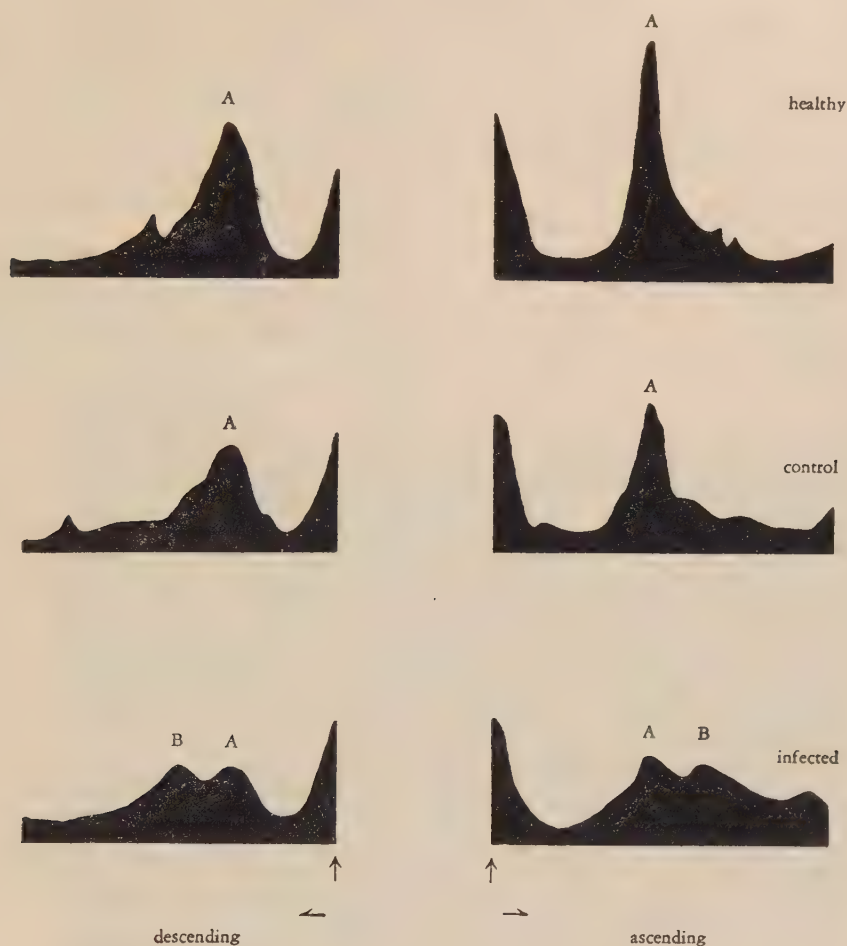4) Uritani, I., and Akazawa, T., J. Agr. Chem. Soc. Japan, 27, 789 (1953)

FIG. 1.   Electrophoretic Patterns.

Descending patterns are those on the left and ascending patterns on the right. 200V, 10mA for 45 min. at 4°C, for each sample.   Mobility values of descending main components $\times 10^5$, cm^2/V-sec. are shown as follows.

	A group	B group
healthy	3.05	—
control	3.77	—
infected	3.29	5.20

like a natural manner[4].   Therefore, it seems that the electrophoretic analyses of Fig. 2 indicate the distinct pattern of the natural protein contained in infected sweet potato, in comparison with the other two sweet potato tissues.

DISCUSSION

It has been reported in our previous paper that there is an increase in the amount of protein contained in the sound part of sweet potato tissue, adjacent to the area infected with *C. fimbriata*[1].   Tha rate of protein synthesis in infected sweet potato is changeable, according to its variety or cultural conditions; generally, a larger increase (a 20–30%-level above the controls) is observable in tissues which contain a higher amount of free amino

O₂ uptake
μ l/μgN
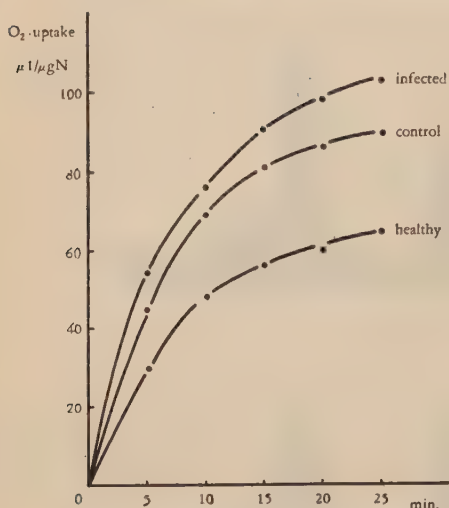
FIG. 2.   Polyphenol Oxidase Activity.

acids, and an increase of about 10–20% is observed in case of sweet potato containing a relatively small amount of amino acids. It is likely that there is not only a net-synthesis of protein constituents from the smaller fragment such as amino acids or amides, but also decomposition and rearrangement of the preexisting protein molecules to form new functional proteins in the infected sweet potato tissues. It is for this reason that we have undertaken the study of the pattern of protein constituents, by electrophoretic analysis.

We may conclude from the data in Fig 1 that a group of protein components marked with B in Fig. 1 is contained in the protein fraction of infected sweet potato, but either scarcely or not, in those of fresh sweet potato and the control tissues. However, at present, it is obscure what kinds of functional proteins have belonged to each group of A and B. Thus, it is highly probable that in sweet potato tissues infected with *C. fimbriata*, the active protein constituents which are distinct from those of the control tissues are synthesized by the condensation of amino acids and/or decomposition and rearrangement of proteins, and these newly formed proteins may in some manner participate in the active metabolism of the infected plant tissues and mechanism of resistance against the invading pathogen.

It is noteworthy, however, that the nitrogen content in the electrophoretic sample of infected sweet potato tissues prepared by Procedure C, was almost the same as that found in the other two groups, notwithstanding the fact that it was higher than the others in the intact tissues. A possible reason for this is that the water-insoluble protein fraction was not extracted in the electrophoretic solution by Procedure C, while the entire $HClO_4$ insoluble fraction was used for protein determination of the intact tissues in the preceding report[1].

Barnett et al[5] have reported that the protein component of wheat leaves infected with rust was distinct from that of healthy leaves when determined by electrophoretic study. Our studies on the black rot of sweet potato along these lines may be considered as another example of the effect of a fungal pathogen having influence on the nitrogen metabolism of host.

SUMMARY

From electrophoretic analysis, it is clarified that the pattern of the protein components in the sound part adjacent to the infected of sweet potato attacked with *C. fimbriata* is different from that of sound sweet potato.

5) Barnett, R.E., and McLaughlin, J.H., *J. Agr. Food Chem.*, **2**, 1026 (1954).

A New Crystalline Germination Promotor for Plant Seeds, Produced by *Streptomyces* sp. S-580

Sir:

It has been already reported by Y. Koaze, H. Sakai and K. Arima[1] that culture broth filtrates and/or mycelial extracts of several microorganisms had promoting effects on the germination of some plant seeds in the filtrate and has succeeded to obtain a crystalline substance which could promote the germination of rice plant seed in such concentrations, as low as 0.1µg/ml. This crystalline substance did not show any antibiotic activity.
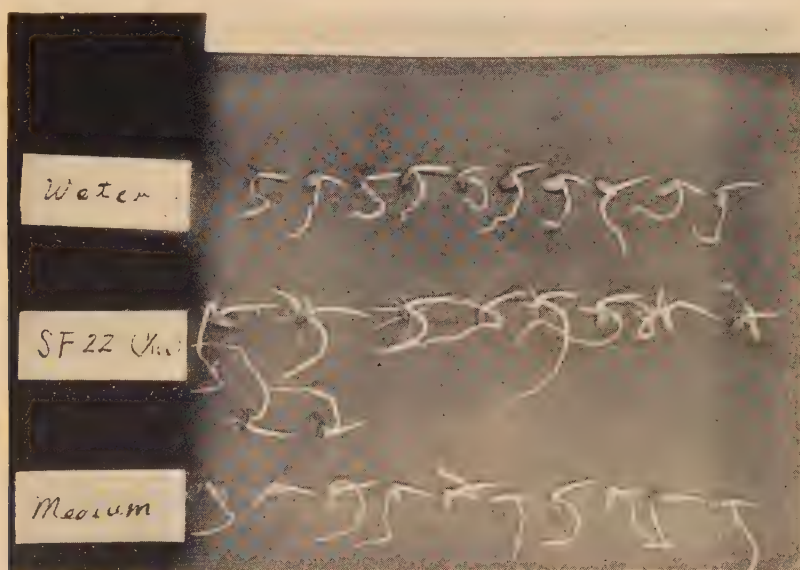
FIG. 1. Effect of the Culture Broth Filtrate of *Streptomyces* sp. S-580 on the Germination of the Rice Plant Seed.

Ten days after start of experiment under the dark condition, incubated at 18°C. Seeds at the middle row treated with the culture broth filtrate of *St.* sp. S-580 diluted to one hundredth. Seeds at the lower row treated with the culture medium of this *Streptomyces* in the same dilution and seeds at the upper row in distilled water, these both being for controls.

standard laboratory seed germination test. Especially, the culture broth filtrate of *Streptomyces* sp. S-580, showed a strong activity having promoting effects on the germination of the rice plant seed as shown in Fig. 1. Therefore, the author attempted to isolate the active substance from this culture broth

A heavy oily syrup with high activity was obtained by extracting the culture broth filtrate of *St.* sp. S-580 with ethyl acetate. By the chromatographic technique using aluminium oxide, an active factor was obtained from the above syrup in crystalline form. (In the above syrup, there were some other active factors which were also isolated.) Repeated crystallizations from absolute ethyl

alcohol gave colorless needles (Fig. 2), m.p. 180–184° (dec.).

This active crystalline substance is a neutral material, soluble in alcohol, benzene, chloroform and water, but insoluble in ethyl ether and petroleum ether. Analytical data of this crystalline substance is as follows:

Anal. Calcd. for $C_{10}H_{16}O_2N_2$: C, 61.20: H, 8.22: N, 14.28: mol wt., 196.24, Found: C, 61.06, 61.18: H, 8.20 8.22: N, 14.05: mol wt.

formula: $C_{10}H_{16}O_2N_2$.

The UV-spectrum of this crystalline substance in methyl alcohol showed no significant absorption in the wavelength range of 221 to 400 mμ but showed a strong end absorption.

The infra-red spectrum in Nujol mull is shown in Fig. 3.

Ninhydrin reaction and biuret reaction were both negative. However, hydrolysis in hydrochloric acid gave the two amino acids,

FIG. 2.   Crystalline Form of this Active Substance. (magnification, ×300).
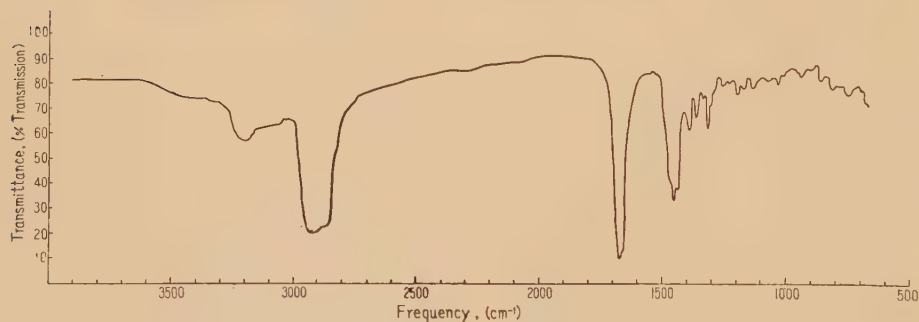
FIG. 3.   Infra-red Spectrum of this Active Crystalline Substance in Nujol Mull.

(by the micro Rast method, dissolved in camphor), 207.19. Therefore, this crystalline substance proved to have the empirical

proline and valine (with regard to their optical configuration studies are now in progress). From above evidences this crys-

talline substance is presumed to be prolyl-valine anhydride

The author wishes to express his sincere thanks to Professor Kin-ichiro Sakaguchi, Univ. of Tokyo for his guidance throughout this work. Thanks are also due to Professor Yusuke Sumiki, Univ. of Tokyo for his kind advice and suggestions. Infra-red analysis was carried out through the courtesy of the Tobacco Research Department, Central Research Institute, Japan Monopoly Corporation.

Yoshihisa KOAZE

Department of Agricultural Chemistry,
Faculty of Agriculture, University of Tokyo

On the Mode of the Conversion of Elymoclavine to Agroclavine*

Sir:

Previously, we[1] found that agroclavine (IV) exclusively yielded a basic substance, lysergine, on treatment with hot sodium butylate.

Recently, we have treated elymoclavine (I) with sodium butylate by a similar procedure as in the case of agroclavine in the hope that a certain derivative corresponding to lysergine would result.

This treatment gave a brown colored mixture from which two basic substances A and B were isolated. A was obtained in a very poor yield; colorless prisms from ethanol, m.p. 249–250° (uncorr. decomp.), $[\alpha]_D^{27} = +50°$, $[\alpha]_{5461}^{27} = +85°$ (c, 0.3 in pyridine). *Anal.* Found: C, 75.68; H, 7.21; N, 10.92. Calcd. for $C_{16}H_{18}ON_2$: C, 75.55; H, 7.14; N, 11.02%. This substance was just identical with lysergol (II) not only in crystal form, melting point and specific rotation, but also in color reaction, paper chromatographic behavior and UV.- and IR.-spectra. B was obtained in an excellent yield; colorless needles or prisms from ethyl acetate, acetone, methanol or ethanol, m.p. 244–245° (uncorr. decomp.), $[\alpha]_D^{20} = +407°$, $[\alpha]_{5461}^{20} = +571°$ (c, 0.18 in CHCl₃), $[\alpha]_D^{20} = +463°$, $[\alpha]_{5461}^{20} = +630°$ (c, 0.23 in pyridine). *Anal.* Found: C, 81.46; H, 7.00; N, 11.69; N-CH₃, 11.94. Calcd. for $C_{16}H_{16}N_2$: C, 81.32;

H, 6.82; N, 11.82; N-CH₃, 12.29%. It gave a yellowish-green color, undistinguishable from that given by molliclavine[2] with Allport and Cocking's reagent, but, unlike the latter, it gave almost no color with concd. sulfuric acid. The UV.-spectrum of this substance was considerably peculiar, showing maxima at the vicinity of 243, 263 and 335 mμ. It was insoluble in petroleum ether, sparingly soluble in benzene, ether, ethyl acetate, acetone, methanol and ethanol, but moderately soluble in chloroform and pyridine. Its solutions in organic solvents exhibited a marked fluorescence. It was almost insoluble in water, but readily soluble in dilute acids. As in both cases of agroclavine and lysergine, this substance exclusively yielded festuclavine (alkaloid "Y") on catalytic hydrogenation, whereas, it yielded the same substance together with agroclavine, lysergine, pyroclavine[3] and costaclavine[3] on reduction with sodium and *n*-butanol. Moreover, this substance itself was obtained in an excellent yield by the treatment of lysergol (II) with hot sodium butylate. These data indicate that this substance possesses the formula III. We have therefore adopted the designation lysergene for this basic product.

Formerly, we[1] found that elymoclavine (I) was converted into agroclavine (IV) by reduc-

CH₂OH

N–CH₃

N
H I

H CH₂OH

N–CH₃

N
H II

CH₂

N–CH₃

N
H III

CH₃

N–CH₃

N
H IV

---
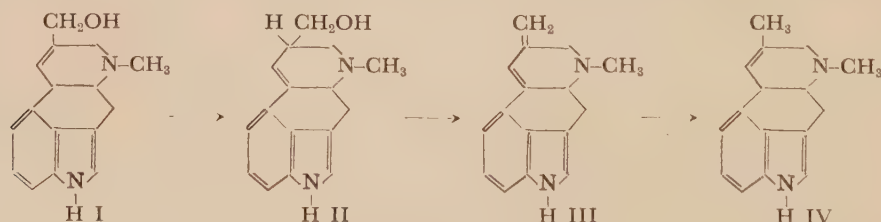
* This work was reported at the Meeting of Agr. Chem. Soc. Japan held at Mie University on October 21, 1956.

1) S. Yamatodani and M. Abe, This Bulletin (communication), **20**, 95 (1956).

2) M. Abe and S. Yamatodani, This Bulletin (communication), **19**, 161 (1955).

3) M. Abe, S. Yamatodani, T. Yamano and M. Kusumoto, This Bulletin (communication), **20**, 59 (1956).

tion with sodium and *n*-butanol. From the results described above, it is evident that the conversion was performed through the process shown here.

Up to the present, lysergol (II) as well as lysergene (III) has not been found either in the sclerotium or in the saprophytic culture of ergot fungus. It is, however, presumed that agroclavine must be produced also in the living cells through the same process as shown above.

Saburō YAMATODANI
Matazō ABE

The Laboratory of the Institute for Fermentation, attached to Takeda Pharmaceutical Industries, Ltd. Osaka, Japan

Further Studies on the Chemical Structure of "Hiochic Acid"

Sir:

In the previous paper[1,2] the author reported the presumed structures of hiochic acid, a new growth factor for *Lactobacillus homohiochii* and *Lactobacillus heterohiochii*. The acid was isolated in crystalline forms, from the culture broth of *Aspergillus oryzae*, as quinine salt (mp. 137–8°) or S-benzylthiuronium salt (122–3°).

The lactone of hiochic acid is a β-hydroxy-δ-lactone having a formula of $C_6H_{10}O_3$, and the following four structures (I–IV) are compatible with the known facts.
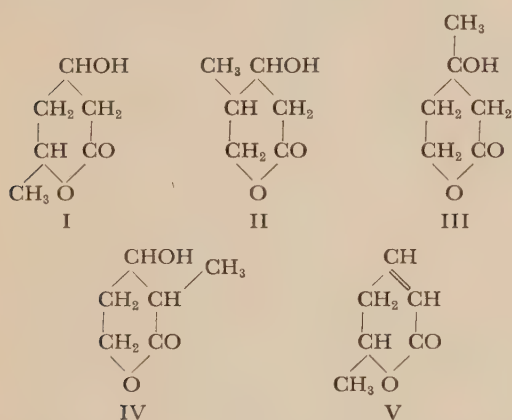
In the previous report[2], it was supposed that β-hydroxy-δ-caprolactone (I) seemed to be most probable, in view of the fact that the iodoform formation test was possitive for natural hiochic acid. However, some corrections of the previous view were necessary from the result of synthesis of hiochic acid and its related compounds.

The α,β-unsaturated acid resulted from hiochic acid by the treatment with 10% H_2SO_4, showed a definite peak of absorption at $222\,m\mu$ ($\varepsilon \doteq 10,000$), while the synthetic

parasorbic acid[3] (V) (b.p. 95–100°/12 mm, $n^{19} = 1.476$) prepared from β,δ-dibromocaproic acid, showed only an end absorption ($\varepsilon \doteq 10,000$ at $208\,m\mu$).

This fact obviously shows that the former is different from parasorbic acid, therefore the presumption of structure I for hiochic acid must be excluded.

As the structure III is also compatible with most of the known characteristics of natural hiochic acid, β-hydroxy-β-methyl-δ-valerolactone (III) was synthesized from β-hydroxy-β-methylglutaric aldehyde by the Tischenko reaction using aluminium isopropoxide. The β-hydroxy-β-methylglutaric aldehyde was prepared from diallylmethylcarbinol. The synthetic lactone thus obtained, was purified by sillica gel chromatography and repeated distillation *in vacuo*.

Anal. Calculated for $C_6H_{10}O_3$: C, 55.37; H, 7.75.
Found: C, 55.51; H, 7.75.

Lactone of natural   b.p. 130–35°/2 mm $n^{19} = 1.474$
    hiochic acid   b.p. 145–50°/5 mm $[\alpha]_D^{20} - 19.9°$ (in
                (bath temp.)   95% cthanol)
Synthetic β-hydroxy   b.p. 145–50°/5 mm $n^{20} = 1.473$
-β-methyl-δ-valero-   (bath temp.)
lactone

The infra red spectra of the synthesized β-hydroxy-β-methyl-δ-valero-lactone both in liquid film and in chloroform solution were entirely identical with natural hiochic acid (Figs. 1 and 2). The iodoform formation from the synthesized lactone with alkaline iodine also proved to be distinctly positive. The biological activity of the lactone for *Lactobacillus heterohiochii* was one half that of natural lactone. From these facts, it is concluded that the lactone of hiochic acid is identical with β-hydroxy-β-methyl-δ-valerolactone and that only the levorotatory isomer of the lactone is biologically active.

During the course of this study, Skeggs

1) Kin-ichiro Sakaguchi; Presented at the Annual Meeting of the Brewer's Association. June 1956. *Annual Report of Brewer's Resarch* **11**, 108, (1956).

2) Gakuzo Tamura, *J. Gener. Appl. Microb.* **2**, 431, (1956).

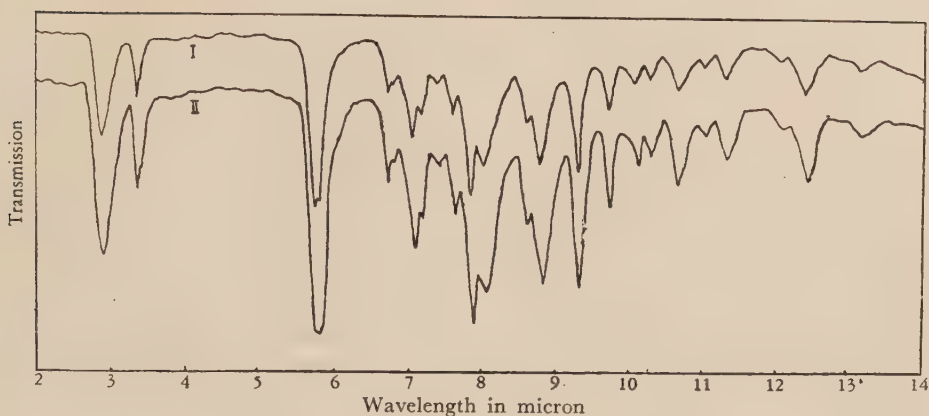3) Richard Kuhn, *Ber. Deut. Chem. Ges.* **76**. 431, (1943).

FIG. 1. Infra Red Spectra in Liquid Film.
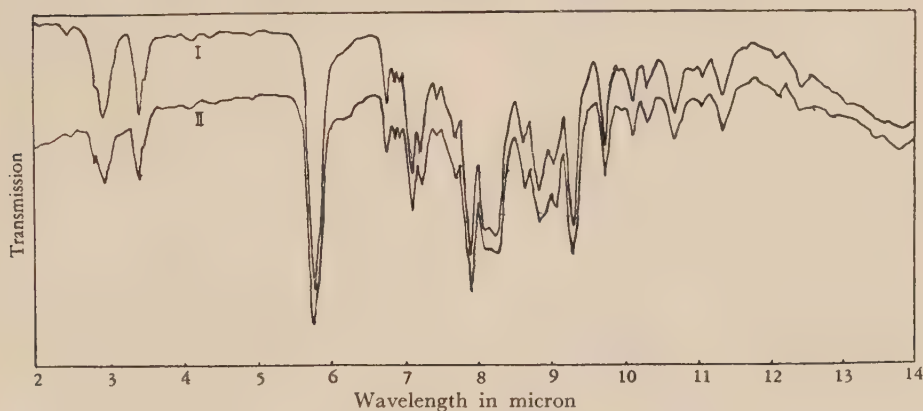I: Synthetic β-hydroxy-β-methyl-δ-valerolactone.   II: Lactone of natural hiochic acid.

FIG. 2. Infra Red Spectra in Chloroform Solution.
I: Synthetic β-hydroxy-β-methyl-δ-valerolactone.   II: Lactone of natural hiochic acid.

et al isolated a new acetate replacing factor for *Lactobacillus acidophilus*[4] and reported that the factor was identified as β-hydroxy-β-methyl-δ-valerolactone[5] (III). They synthesized this lactone by partial reduction of β-hydroxy-β-methylglutaric acid and named it divalonic acid. The iodoform formation test of their factor was reported to be negative. However, it was confirmed by the present author that the synthetic divalonic acid, which was kindly supplied from Dr. K. Folkers, was also biologically active for the growth of our test organism, *Lact. heterohiochii* which does absolutely require hiochic acid. The activity of synthetic divalonic acid was also one half that of natural hiochic acid as likely as was the lactone synthesized by the present author. It is apparent, therefore, that the chemical structure of hiochic acid is identical with that of divalonic acid.

The author wishes to express his sincere thanks to Prof. Kin-ichiro Sakaguchi for his guidance throughout this work, and to Dr. Karl Folkers, Merck Sharp and Dohme Research Laboratories, for the salt of divalonic acid so generously supplied by him. Thanks are also due to Prof. Masanao Matsui, Prof. Kyosuke Tsuda and Assis. Prof. Saburo Tamura for their kind advice and suggestions.

Gakuzo TAMURA

Department of Agricultural Chemistry,
Faculty of Agriculture, University of Tokyo
Received April 5, 1957

4) Helen R. Skeggs et al.,*J. Bact.* **72**, 519 (1956).
5) Donald E. Wolf et al., *J. Am. Chem. Soc.* **78**, 4499, (1956).

Microdetermination of Parathion

Sir :

In the research for sea and river water contamination and damages of marine organisms caused by insecticides, there is a keen need for highly sensitive and specific methods for the determination of insecticides, especially parathion on account of its great toxicity. Several published spectrophotometric methods for parathion determination are based on the absorbances of parathion itself($\lambda_{max.}$ 274 mμ, ε 9.0$\times$10^3),[1][2] and p-nitrophenate ion ($\lambda_{max.}$ 405 mμ, ε 2.1$\times$10^4)[3][4] and on the color of azo dyes ($\lambda_{max.}$ 560 mμ, ε 4.7$\times$10^4 [5]) and $\lambda_{max.}$ 510 mμ, ε 1.7$\times$10^4 [6]).

From the results of the preliminary tests conducted in this laboratory for phosphorus compound determination, the method of Soyenkoff ($\lambda_{max.}$ 510 mμ, ε 6.1$\times$10^4)[7] and its modified one of this laboratory ($\lambda_{max.}$ 480 mμ, ε 1.5$\times$10^5) have been employed. As the results, the following two procedures have been designed as the highly sensitive and specific methods for the estimation of small amounts of parathion which are contained in water. The basic steps of Method 1 for the concentration order of p.p.h.m. or more are : (1) Extraction, (2) reduction, (3) development and evaluation of an azo dye color, (4) digestion of an azo dye, (5) estimation of phosphorus. Those of Method 2 for the concentration order of p.p.b. are : (1) Extraction, (2) condensation and feeding on a paper strip, (3) paper chromrtography with non-aqueous solvent system, (4) elution of the spot, (5) digestion of paration, (6) colori-metry of phosphorus.

Procedure : Method 1—One liter of the sample is added with 17 ml of concentrated hydrochloric acid and 50 g of sodium chloride, and extracted one time with each of 100 ml and then 50 ml portions of n-hexane, using a separatory funnel†. The combined hexane layer is filtered and evaporated just to dryness with a modified Kohn's rotating evaporator[8] or an agitating evaporator designed in this laboratory. To the residue are added 10 ml of glacial acetic acid, 5 drops of concentrated hydrochloric acid and 0.3 g of zinc dust, and the mixture is gently boiled for 10 minutes. After cooling, it is filtered with suction, then the residue washed with 3 ml of glacial acetic acid. The combined filtrate is cooled and added with 0.5 ml of 0.5% sodium nitrite, 0.5 ml of 5% ammonium sulfamate and 2 ml of 1% N-(1-naphthyl)-ethylenediamine hydrochloride solution mixing them well in this order with each 15-minute interval under cooling at about 4°. The colored solution is made up to 20 ml with glacial acetic acid and kept at about 25°. After standing for 30 to 60 minutes, the color at 560 mμ is measured. Then, the azo dye solution transferred to an Erlenmyer flask is evaporated to about 10 ml with flowing air immersing the flask partially in a water bath maintained at 95°. The condensate is evaporated to dryness in a digestion tube (20$\times$250 mm, borosilicate glass) and digested with 10 drops of concentrated sulfuric acid and 7 drops of 30% hydrogen peroxide at 155° (oil bath) for 80 minutes. At the end of digestion, each tube is added with 5 ml of water, heated in a boiling water bath for 10 minutes, neutralized, then made

1) American Cyanamide Company, Agricultural Chemicals Division: Manual of Manufacturing Information for Thiophos (1952).

2) M. Kotakemori, *Ann. Takamine Lab., Japan*, **6**, 146 (1954).

3) J.A.A. Ketelaar, and J. E. Hellingman, *Anal. Chem*, **23**, 646 (1951).

4) K. O'Keefe, and P.R. Averell, *ibid.* **23**, 1167 (1951).

5) P.R. Averell, and M.V. Norris, *ibid.* **20**, 753 (1948).

6) J.C. Gage, *Analyst*, **75**, 189 (1950).

7) B.C, Soyenkoff, *Biol. Chem.*, **198**, 221 (1952).

† If necessary, 5ml. of 10% alum solution should be added to precipitate the impurities.

8) P. Kohn,: *Anal. Chem.*, **28**, 1061 (1956).

up to 10 ml. A two-ml aliquot of this solution is added with 2 ml of the dye solution (ethosulfate of 2-*p*-dimethylaminostyrylquinoline), then 1 ml of the molybdate-sulfate solution, according to the method of Soyenkoff.

The optical density at 510 mμ is read after 10 minutes. The two colorimetric data of the sample, absorbances of the azo dye and phosphorus color complex, are corrected by those of the blanks, respectively. The estimates of the paration content obtained by the aid of each standard curve from the azo dye and phosphorus colorimetries can be ascertained by the agreement of these two values. If these were discrepancy between them, the lower value should be chosen as the upper limit of the paration content.
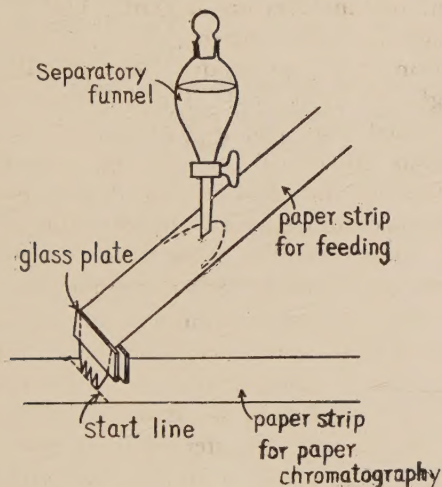
FIG. 1. Total Sample Feeder for Paper Chromatography.

Method 2—About 15 ml of the condensate of the hexane layer obtained from 1 liter of the sample by the same treatment as Method 1 are fed on a start line of a paper strip for paper chromatography. A total sample feeder for paper chromatography illustrated in Fig. 1 serves for feeding within the narrow area on the paper strip. As for paper chromatographic techniques, the descending method with methanol-hexane (1 : 2 v/v)

TABLE I. R_F VALUES OF PARATHION AND RELATED COMPOUNDS

Compound	R_F
Parathion, laboratory	0.74
Parathion, commercial	0.07, 0.24, 0.33, 0.71
Emulsifier of Parathion	0.02*
Methylparathion	0.57
Chlorthion	0.63
Ethylchlorthion	0.80
4124 (isochlorthion)	0.66
Ethyl 4124	0.84
O-(2-nitrophenyl)-O, O-diethyl thiophosphate**	0.24, 0.35, 0.63, 0.71
Malathion	0.02*
Diazinon	0.01*
TEPP	0.05*
Adenosinetriphosphate	0*
Ethylthiophosphate	0.01*
Methylthiophosphate	0*
Orthophosphoric acid	0*
Pyrophosphoric acid	0*

mobile phase, hexane layer; stationary phase, methanol layer development of spot :
　　　* sprayed FeCl$_3$ and sulfosalicylic acid reagents; the others, alcoholic potassium hydroxide.
　　** decomposed sample

system is employed††. The R_F value of pure parathion is 0.7–0.8, and a sample of commercial parathion gives four spots developed by alcoholic potassium hydroxide with a yellow color which is due to a *p*-nitrophenate ion. The spot of parathion cut off by guiding of its authentic sample is eluted with the descending flow of about 10 ml of acetone. The eluate is evapolated to dryness. The residue is digested with 3 drops of concentrated sulfuric acid and 2 drops of 30% hydrogenperoxide containing no phosphorus compound as the stabilizer at 155° for 80 minutes in the oil bath. The tube-wall should be carefully rinsed with the digestion reagent by rotating the tube in advance of digestion. At the end of digestion, each tube is cooled, added with 0.5 ml of diluted perchloric acid (2.5 ml of 60% HClO$_4$+50 ml of

†† Metcalf and March[9] have reported a procedure involving reversed phase paper chromatography and colorimetry of phosphorus, but their method is not practical in this case.

9) R.L. Metcalf, and R.B. March, *Science* 117, 527 (1953).

water), and then neutralized by 5 N sodium hydroxide using the phenolphthaleine solution as an indicator. After acidifying with 1 drop of 0.5 N sulfuric acid, the solution is made up to 2 ml. Two ml of the dye solution and then 1 ml of the molybdate-sulfate solution are added under agitation. The

TABLE II. ANALYTICAL DATA

	Amount of Parathion in 1 l of sample (γ)		Mean of four Absorbances	Coefficient of Variation(%)	Recovery (%)
Method 1	60	azo dye	0.481 (at 560 mμ)	0.8	98.8
		phosphorus	0.160 (at 510 mμ)	2.4	99.0
Method 2	4		0.432 (at 480 mμ)	2.8	89.0

optical density at 480 mμ is read exactly 5 minutes after the addition of the last reagent against a blank from 1 liter of water similarly treated. The parathion content of the sample is obtained by referring the absorbancy at 480 mμ to a calibration curve.

The analytical data are shown in Table II. The results concerning the above two methods will be reported in detail later.

Yusuke SUMIKI
Akira MATSUYAMA
Hiromichi KATO
Yushih CHEN
Teiji YAMAUCHI

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo